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붉은 곰팡이에 감염하는 바이러스의 분자 생물학적 특성 및 방어 반응 관련 기주 요인의 특성 구명

Characterization of Fusarium viruses and host factor *FgHal2* required for host defense against Fusarium graminearum virus 1 infection

2015년 2월

서울대학교 대학원

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Characterization of Fusarium viruses and host factor *FgHal2* required for host defense against Fusarium graminearum virus 1 infection

A dissertation submitted in partial
fulfillment of the requirement for
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JISUK YU

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A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Characterization of Fusarium viruses and host factor *FgHal2* required for host defense against Fusarium graminearum virus 1 infection

UNDER THE DIRECTION OF DR. KOOK-HYUNG KIM

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

BY
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
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Characterization of Fusarium viruses and host factor *FgHal2* required for host defense against Fusarium graminearum virus 1 infection

JISUK YU

ABSTRACT

Many mycoviruses have been identified from diverse plant-pathogenic *Fusarium* species including *F. graminearum*. Infections with mycoviruses in *F. graminearum* mostly remain persistently and asymptomatically in their host, however, some mycoviruses causing altered phenotype, such as reduced growth, pigmentation, sporulation or virulence. Among the Fusarium graminearum viruses (FgVs), FgV1 or FgV2 infections caused several phenotypic alterations in *F. graminearum.* In contrast, FgV3 and FgV4 infections did not cause any phenotypic change. In this study, we determined complete genome sequences of FgV2, FgV3, and FgV4 and analyzed its phylogenetic relationship with other mycoviruses. The three mycoviruses consist of one to five different segments of dsRNA ranging in

size from approximately 1.7 to 9.3 kb. FgV2 consist moiinocistronic dsRNA segments, denoted as dsRNA-1 to dsRNA-5. The lengths of FgV2 dsRNAs 1–5 ranged from 2414 to 3580 base pairs (bp). The viral genome of FgV3 is 9098 bp long and contains open reading frame (ORF) encoding a putative RNA-dependent RNA polymerase and a protein of unknown function. The FgV4 genome is composed of two dsRNA genome segments of 2383bp and 1739bp. According to phylogenetic analysis, the FgV2, FgV3, and FgV4 is closed to *Chrysoviridae*, *Totiviridae*, and *Partitiviridae*, respectively, but consist a distinct virus clade within the Family.

Transcriptomic and protein expression profiling have shown that many *F. graminearum* genes are differentially expressed as a consequence of FgVs infection. Of the identified host genes involved in the interaction between mycovirus and fungus host, *FgHal2* shows reduced expression in response to FgV1 infection. *FgHal2* has a highly conserved 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) domain or inositol monophosphatase (IMPase) superfamily domain. We generated targeted gene deletion and over-expression mutants to clarify the role(s) of *FgHal2* in FgV1 infection. The *FgHal2* deletion led to decrease in mycelial growth, aerial mycelia formation, and pigmentation whereas over-expression mutants were morphologically similar to the wild type (WT). Furthermore, compared to the

WT, the gene deletion mutant produced fewer conidia and these showed abnormal morphology. The *FgHal2* expression level was decreased by FgV1 infection at 120 h post-inoculation (hpi) whereas the levels were 9-fold greater for both the virus-free and virus-infected over-expression mutant than for the WT. FgV1 RNA accumulation was decreased in the deletion mutant at 48, 72, and 120 hpi. FgV1 RNA accumulation in the over-expression mutant was reduced relative to the WT at 48 and 72 hpi but was similar to that of the WT at 72 hpi. Furthermore, the low vertical transmission rate of FgV1 and continually growing of virus-free sectors in the colonies of FgV1-infected gene deletion mutant suggesting that *FgHal2* might be required for stable maintenance of FgV1 infection. Together, these results indicate that the putative 3'(2'), 5'-bisphosphate nucleotidase gene, *FgHal2*, has diverse biological functions in the host fungus and might affect the viral RNA accumulation and transmission of FgV1.

KEY WORDS: Fusarium graminearum virus, mycovirus, *Fusarium graminearum*, *FgHal2*, 3'(2'),5'-bisphosphate nucleotidase, secondary metabolism, virus–host interaction

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GENERAL INTRODUCTION

Mycoviruses are widely distributed in various fungi including mushrooms and plant pathogenic fungi, and usually many of them are persistently infect their hosts without extracellular phase (Nuss, 2011; Xie and Jiang, 2014). Mycoviruses are transmitted by hyphal anastomosis or through asexual and/or sexual spores because extracellular phase is absent (Nuss, 2011). Mycoviruses are generally grouped into several families, including *Totiviridae*, *Partitiviridae*, *Chrysoviridae*, *Hypoviridiae*, and *Nanoviridae* (Xie et al., 2014). Mycoviruses have been suggested as a biological-control agent for plant fungal disease and useful tools for exploring fungal biology and virology. Most of mycoviruses present in their host relatively unnoticed, however, some of fungal viruses cause altered host phenotype, such as reduced growth, pigmentation, sporulation or virulence (Nuss, 2011). These mycoviruses which attenuate fungal virulence and cause physiological change have been studied in plant pathogenic fungi including *Botrytis cinerea*, *Cryphonectria parasitica*, *Ophiostoma ulmi*, *Rosellinia mecatrix*, and *Fusarium gramineraum* (Göker, 2011).

Fusarium graminearum is a homothallic ascomycetes fungus that causes Fusarium head blight (FHB) of wheat, barley, and rice and ear and stalk rot

in maize (Leslie, 2006). In addition to reducing yield and quality of crops, this pathogen produces mycotoxins such as trichothecenes and zearalenone, which are very harmful to humans and other animals (Desjardins, 2006).

Several mycoviruses were identified in *Fusarium* species including *F. graminearum* (Cho et al., 2013). Previously, various patterns of dsRNAs were presented in *F. graminearum* which isolated from infected maize and barley in Korea (Chu et al., 2004). Among the Fusarium graminearum viruses, Fusarium graminearum virus 1 strain DK21 (currently named FgV1) has been identified firstly. FgV1 was reported that it associated with reduced host virulence, a reduced mycelial growth, increased pigmentation, and inhibition of the mycotoxin production (Chu et al., 2002). FgV1-infected fungal strain observed severe symptom, whereas the other dsRNA-contained *F. graminearum* strain show moderate symptom or asymptomatic phenotype (Chu et al., 2004). This data indicated the genomic diversity in dsRNA mycoviruses that infect *F. graminearum*.

FgV1 presents as double-stranded RNA forms and the genome consists of 6,624 nucleotides, carries a poly (A) tail at 3′-terminal. The viral genome has four putative open reading frames (ORFs) and 5' (53 nt) and 3' (46 nt)-untranslated regions (UTRs) (Kwon et al., 2009b). Also, two subgenomic RNAs encode ORF2, ORF3, and ORF4. The deduced amino acid sequence

of ORF1, which encodes a putative RNA-dependent RNA polymerase (RdRp) but ORFs 2-4 have no sequence homology with known protein (Kwon et al., 2009b). FgV1 is phylogenetically closely related with *Hypoviruses* but resemble a potex-like viruses in genome organization and sgRNA accumulation (Kwon et al., 2009b). Therefore, advances in our understanding of the functions of viral gene that responsible for the hypovirulence trait of the FgV1 is needed to provide new insight into the relationship between host and viral elements. In addition, characterization of host cellular proteins which involved in all steps of virus life cycles is required for further study.

Viral genomes interact with numerous cellular components to accomplish intracellular multiplication of viruses (Whitman and Wang, 2004). Some of the interactions between viral and host factors contribute to the efficient viral replication and movement. These interactions are also related with modulation of host gene expression and defense responses against the viral infection (Whitman and Wang, 2006). Interaction study between the hypovirulence-associated virus and its host provide an opportunity to characterize viral determinants that are responsible for an altered phenotypes of fungal host and to understand the molecular basis of fungal biology (Xie and Jiang, 2014). However, we still know very little about which host protein is involved and how mycoviruses interact with host proteins to mediate virus

replication or to induce disease resistance.

Previous researches demonstrated that mycovirus infections globally affect the expression of host genes and cause hypovirulence or phenotypic alterations in the fungal host (McBride et al., 2013; Allen and Nuss, 2004). Several host genes regulated by *Cryphonectria hypovirus* 1 strain EP713 (CHV1) infection have been identified in the plant-pathogenic fungus *Cryphonectria parasitica*. Some host genes down-regulated by CHV1 represented a broad range of biological function, such as female fertility, conidiation, virulence, stress response, and transmission of the hypovirus in *C. parasitica* (Gao et al., 2013; Park et al., 2004; Sun et al., 2009).

Based on transcriptomic and proteomic analysis, we previously determined that many fungal host genes are differentially expressed in response to FgV1 infection. These host genes are involved in the cell signal transduction, metabolic pathways, stress response, post-transcriptional gene silencing, differentiation, and other processes (Kwon et al., 2009a; Cho et al., 2012; Lee et al., 2014). These results indicate that some of those genes might function as host factors for maintaining the life cycle of FgV1 (Kwon et al., 2009a; Son et al., 2013). For example, *Hex1*, essential component of the Woronin body, maintain cellular integrity and pathogenicity of *F. graminearum* (Son et al., 2013). Moreover, the accumulation of FgV1 viral

RNA depends on the *HEX1* accumulation, although the molecular mechanisms underlying Hex1 proteins-FgV1 interactions are not clarified yet (Son et al., 2013). Other host genes in *F. gramine*arum that may directly and/or indirectly affect the life cycle of FgVs, however, remain poorly understood.

In this study, interaction between specific host protein and FgV1 were investigated to verify the host cellular functions and the role(s) of this interaction during the FgV1 infection. In addition, the whole genome sequences of FgV2, FgV3, and FgV4 were revealed for further investigation of characterizing viral element which related to viral symptom expression or antiviral defense response.

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CHAPTER I

Molecular characterization of

Fusarium graminearum virus 2-4 isolated from

Fusarium graminearum strain 98-8-60 and DK3

ABSTRACT

The complete genomes of three different dsRNA mycoviruses, Fusarium graminearum virus 2 (FgV2), Fusarium graminearum virus 3 (FgV3), and Fusarium graminearum virus 4 (FgV4), was sequenced and its phylogenetic relationship with other mycoviruses was analyzed. FgV2 infects *Fusarium graminearum* strain 98-8-60 and consist monocistronic dsRNA segments, denoted as dsRNA-1 to dsRNA-5. The lengths of FgV2 dsRNAs 1–5 ranged from 2414 to 3580 base pairs (bp). FgV2 dsRNA-1 encodes RNA-dependent RNA polymerase (RdRp) containing eight conserved motifs. The 5' and 3' untranslated regions (UTRs) are highly conserved, and each dsRNA segment had 78–105 and 84–306 bp of 5' and 3' UTRs, respectively. The viral genome of FgV3 is 9098 bp long and contains ORF encoding a putative RdRp and a protein of unknown function. The FgV4 genome is composed of two dsRNA genome segments of 2383bp and 1739bp. FgV4 dsRNA-1 contains a single ORF which has a conserved RdRp motif while FgV4 dsRNA-2 contains two putative ORFs coding for products of unknown function. Phylogenetic analysis with other dsRNA mycoviruses of the putative RdRp protein, FgV2 consist a distinct virus clade with Aspergillus mycovirus 1816 (AsV 1816) and Magnaporthe oryzae chrysovirus 1 (MoCV1) in the family *Chrysoviridae*. However, genome organization and phylogenetic analyses indicated that

FgV3 was related to the *Totiviriridae* and *Chrysoviridae* families and FgV4 formed a distinct clade in the family *Partitiviridae.*

INTRODUCTION

The fungus *Fusarium graminearum* is a plant pathogen that causes head and seedling blight of small grains such as wheat and barley, stalk and ear rot of corn, and stem rot of carnation thus cause enormous economic losses and mycotoxin contamination in cereals (Cook, 1981; Lee et al., 2010).

In previous research, double-stranded RNAs from several *F. graminearum* strains were determined the diversity and incidence of mycoviruses that infect *F. graminearum* clade (Chu et al., 2004) (Fig 1). Among these dsRNAs-contained fungal strains, Fusarium graminearum virus 1–DK21 (FgV1) has been reported to be associated to differing degrees with several hypovirulence-associated parameters, including reduced mycelial growth, increased red pigmentation, and reduced virulence on wheat plants (Chu et al., 2004; Kwon et al., 2007a). This hypovirulence-associated phenotype is unique feature of FgV1-infected strain compare with other dsRNA-infected *F. graminearum* strains. The morphological phenotype of FgV2-infected fungal stains has hypovirulence-related symptom but not causes severe change in fungal host. Moreover, no association with any distinct morphological or pathogenicity phenotypes was observed in other

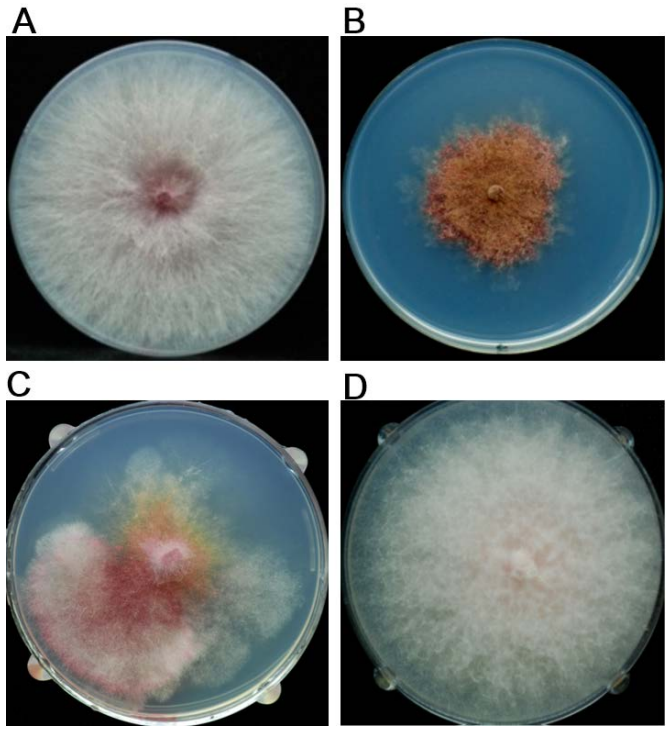

Fig. 1. Morphology of FgVs-infected F. graminearum strains. (A) virus-free *F. grmainearum* DK21 strain. (B) FgV1-infected *F. grmainearum* DK21 strain (C) FgV2 infected *F. grmainearum* 98-8-60 strain (D) FgV3 and FgV4 infected *F. grmainearum* DK3 strain. All strains were grown at 25℃ for 5 days on PDA media.

dsRNA-containing *F. graminearum* isolates including *F. graminearum* strain DK3, indicating that this dsRNA mycoviruses infect *F. graminearum* asymptomatically (Yu et al., 2009).

Complete genome sequence analysis and molecular characterization of FgV1-DK21 indicated that genomic organization and gene expression strategy are similar to those of the plant potex-like ssRNA virus group (Kwon et al., 2007). The other three mycoviruses (FgV2, FgV3, and FgV4) have not yet been characterized in detail. Additionally, the Fusarium graminearum virus- china9 (FgV-ch9) was identified that this virus is closely related to FgV2 based on partial RdRp sequences, related hypovirulence phenotypes in *F. graminearum* (Darissa et al., 2011).

In the present study, the nucleotide sequences of three different dsRNA viruses, Fusarium graminearum virus 2 (FgV2), Fusarium graminearum virus 3 (FgV3) and Fusarium graminearum virus 4 (FgV4) were determined. Phylogenetic analysis of deduced amino acid sequences of putative RNA-dependent RNA polymerase (RdRp) revealed that FgV2 forms a new cluster within the family *Chrysoviridae*, FgV3 was closely related to members of the families *Totiviridae* and *Chrysoviridae*, whereas FgV4 was found to form a distinct virus clade within the family *Partitiviridae*.

MATERIALS AND METHODS

I. Fungal strain and culture conditions

All strains used were stored in 15% (v/v) glycerol at -80℃ and were reactivated on Difco potato dextrose agar (PDA) (BD, New Jersey, U.S.A.). Fungal strains used for extractions of total RNA were grown in 50 ml of liquid complete medium (CM) at 25℃ at 150 rpm for 5 days. Mycelia were collected by filtration through Whatman 3MM filter paper (GE Healthcare, Uppsala, Sweden), washed with distilled water, pressed between paper towels to remove the excess water, and frozen at -80℃.

II. Double-strand RNA extraction

Frozen mycelia were ground by using liquid nitrogen and with a mortar and pestle. Total RNAs were extracted with Iso-RNA Lysis reagent (5 PRIME, Gaithersburg, USA) according to the manufacturer's protocol. The dsRNAs from total RNAs of fungal strains were purified through a Whatman CF11 cellulose chromatography (GE Healthcare) as described previously with some modifications (Chu et al, 2002). Briefly, after the extracted total RNA were suspended in 1X STE (0.05M Tris-HCl, 0.1M NaCl and 1 mM EDTA, pH 7.0) and ethanol was added to a final concentration of 17% (v/v). The mixture was

loaded and flow through to 15 ml syringe with 1g of CF-11 cellulose. The column was washed with 40 ml of 1x STE containing 17% ethanol (v/v), the bound dsRNA was eluted with 10 ml of 1x STE buffer and precipitated with same volume of isopropanol for 2 h at -20°C. The dsRNA pellet was collected by centrifugation, washed with 80% (v/v) ethanol, dried and suspended in DEPC-treated water and followed by treatment with DNase I (Takara Bio Inc. Japan) to remove genomic DNA completely. The final purified samples were precipitated with ethanol, and finally suspended in DEPC-treated water. Each dsRNAs were separated on a 1.5% agarose gel, and visualized on a UV transilluminator after ethidium bromide (EtBr) straining.

III. cDNA synthesis, amplification, cloning

Purified dsRNAs were sheared using a nebulizer (Invitrogen) to generate fragments having a length of smaller than 2kb. cDNAs were synthesized and amplified using tagged random primers (5'-ACTAGCCATGCAGCCCTT NNNNNNNN-3'). The cDNAs were amplified by using adaptor sequence primer (5'-ACTAGCCATGCAGCCCTT-3'). The amplified cDNA products were cloned into pGEM T-easy vector (Promega) for sequencing. Every base was determined by sequencing at least three independent overlapping clones in both orientations. Nucleotide sequence data were assembled using the

Seqman program (DNASTAR, Inc. USA). Based on the DNA sequences obtained, dsRNA-specific primers were designed and used for RT-PCR.

IV. 5' and 3' RACE

The 5' and 3' ends cDNA amplifications were performed using a slightly modified classical RACE protocol (Cold spring Harbor Laboratory Press, 2005). Briefly, synthesis cDNA using gene specific primers which located closed to 5' and 3' end of region. After remove excess primers, treated with terminal transferase and deoxycytidine triphosphate (dCTP) to generate dC-tailed cDNA. Using adaptor primer-5'-CGTCGAAGCTTGAATTCT-3' and $(dG)_{17}$- adaptor primer-5'-CGTCGAAGCTTGAATTCT$(G)_{17}$-3', PCR reaction first consist 5 cycle of 94°C for 1 min, 50°C for 5 min, and 72°C for 5 min followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. After the cycles were completed, the reaction mixtures were held at 72°C for 10 min and then at 4°C. PCR products were purified and ligated into pGEM T-easy vector (Promega, USA) for sequencing. Nucleotide sequence data were assembled using the Seqman program (DNASTAR, Inc. USA). Compatible terminal sequences were determined by at least three independent overlapping clones in both orientations.

V. Northern blot hybridization

Northern blot hybridization was conducted as described previously (Chu et al, 2002). Each dsRNA segment was identified by Northern hybridization using the cloned cDNAs as probes. Five DNA probes for FgV2, P1-P5 specific for dsRNAs 1-5 respectively (P1: 2371-2774, P2: 747-1284, P3: 972-1383, P4: 1152-1512, P5: 203-682). ^{32}P-labeled DNA probes corresponding to the internal region of each dsRNA segment were prepared as previously described (Chu et al, 2002). The hybridized membranes were exposed to BAS imaging plate (Fujifilm, Japan) and visualized using a Fujix BAS 2500 bioimage analyzer (Fujifilm, Japan). Blots were stripped in boiling stripping solution (0.1× SSC-0.1% SDS) for 10 min and were reprobed several time.

VI. Sequence alignment and phylogenetic analysis

A sequence similarity search of viral RNAs was conducted with the NCBI BLAST program. The alignment of putative RdRp amino acid sequences of FgV2-4 and selected dsRNA mycoviruses was performed using BioEdit sequence alignment editor (Version 7.0.9) (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and GeneDoc programs (http://www.nrbsc.org/gfx/genedoc/). On the basis of the aligned sequences, phylogenetic trees of RdRp regions of dsRNA mycoviruses including FgVs were constructed using

the neighbor-joining method on MEGA version 4.0 program (Kumar et al., 2008).

RESULTS

I. Genome organization of FgV2

In the current study, at least five dsRNA segments were observed in purified dsRNAs of FgV2 when separated on a 6% polyacrylamide gel (data not shown). One or two additional dsRNAs were infrequently observed in both repeated subculturing and single spore isolation. It is possible that these dsRNAs may be satellite RNAs, and thus they were not regarded as genome segments of FgV2. The five dsRNAs in *F. graminearum* strain 98-8-60 were designated as dsRNA-1 to dsRNA-5 that consistently detected. The length of dsRNAs 1–5 ranged from 3580 to 2414 bp (Fig. 2).

The 5' UTR of these dsRNA segments were 78–105nt. Comparison of the 5' UTR sequences of all dsRNA segments revealed a stretch of conserved sequences including GCAAAAAAGA at the 5' terminus of each dsRNA segment (Fig. 3A). Sequences in the 5`UTR was highly conserved in the region downstream of the ATG initiator codon. The CAA repeats, characteristics of the 5`UTR of *chrysoviruses* (Jiang and Ghabrial, 2004) were observed in FgV2, but low frequency compare to those of other *chrysovirus* such as PcV and CCRS-CV (Cherry chlorotic rusty spot crysovirus). The similar repeats in 5`UTR region were known as translational

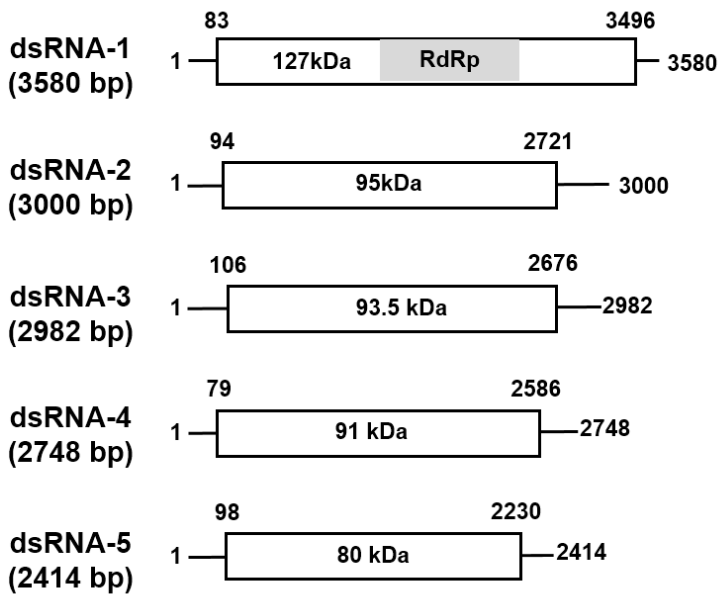

Fig. 2. Genome organization of FgV2. The rectangular box represents the ORF defined by sequence analysis, and the molecular weights of the proteins encoded by each ORF are indicated in the boxes. The grey bar in the center of the dsRNA-1 ORF represents the conserved viral RNA-dependent RNA polymerase (RdRp) domain. The un-translated regions are indicated by a single line. The nucleotide positions of the initiation and termination codons are indicated above the border of the ORF.

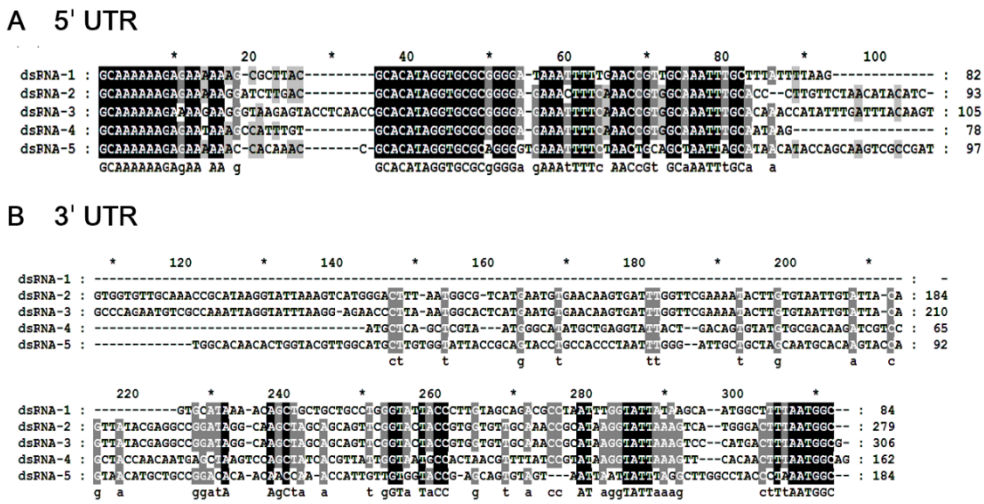

Fig. 3. Conserved sequence elements in FgV2 dsRNA segments. Sequences of the 5′ UTR (A) and 3′ UTR (B) of each dsRNA segment that are identical are reverse highlighted. Black, dark grey, and light grey shading levels represent 100%, 80%, and 60% nucleotides conservation, respectively.

enhancer elements of *tobamoviruses* (Gallie and Walbot, 1992). The 3' UTR of the five genomic dsRNAs also contained conserved sequences (Fig. 3B). The 3`UTRs of FgV2 are relatively long, between 84–306 nt in length. In addition, terminal sequences of 3`UTRs of FgV2 showed almost identical among the five dsRNA segments.

The ^{32}P-dCTP labeled DNA fragment corresponding to each dsRNA segments were used as probes for Northern blot analysis to determine sequence similarity and to detect the presence of virus-specific RNAs in infected cells. Each probe specifically hybridized to each dsRNA in FgV2 infected *F. graminearum* (Fig. 4). This result indicates that each segment contains unique sequences. The complete nucleotide sequences of dsRNA segments 1 to 5 of FgV2 were deposited in GenBank (accession numbers HQ343295 to HQ343299, respectively).

Computer analysis of FgV2 dsRNAs 1–5 showed that each dsRNA contained a single ORF encoding 127-, 95-, 93.5-, 91-, and 80-kDa proteins, respectively (Fig. 2). FgV2 dsRNA-1 contain 8 conserved RdRP motifs and conserved RdRp domain which has sequence similarity with RdRp of other mycoviruses (Fig. 5). FgV2 dsRNA-2 does not contain a specific domain expecting a function but the deduced amino acid sequence of FgV2 dsRNA-2 showed relatively high sequence identity (29%) with MoCV1 dsRNA

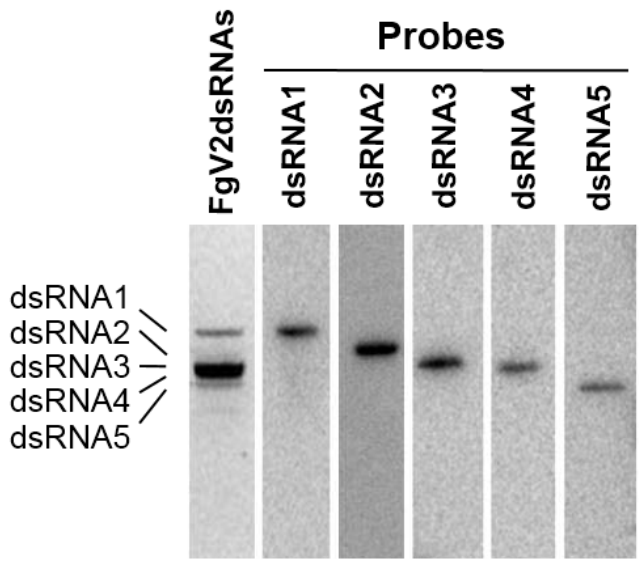

Fig. 4. Northern blot analysis. Purified dsRNAs were separated on a 1% agarose gel, stained with ethidium bromide, and blotted to Hybond-N+ membrane. ^{32}P-dCTP labeled DNA probes specific for each dsRNA region (nt 1257–1674, 747–1284, 2339–2766, 1152–1512, and 203–682 of dsRNAs 1 to 5, respectively) were prepared and used for hybridization. The membrane was reused for the blot to detect different segments of FgV2 by using multiple probes. Each of the dsRNA specific probes was stripped completely before rehybridizing with another probe.

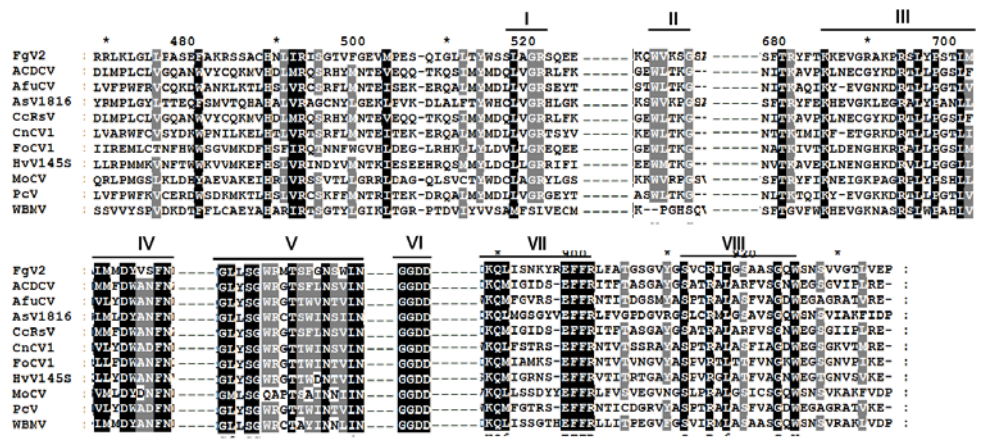

Fig. 5. Amino acid sequence alignment including eight conserved RdRp motifs of FgV2 with those of other dsRNA mycoviruses in the Chrysoviridae family. Numbers I-VIII indicate the eight conserved motifs in RdRps. The positions of the amino acid sequences are indicated on the right. Abbreviations of virus name and Genbank accession numbers are the following: AfuCV, Aspergillus fumigatus chrysovirus (FN178512); AsV1816, Aspergillus mycovirus 1816 (EU289896); CCRSV, Cherry chlorotic rusty spot chrysovirus (AJ781397); FgV2, Fusarium graminearum virus 2 (HQ343295); FgV-ch9, Fusarium graminearum mycovirus-China 9 (HQ228213); FoCV1, Fusarium oxysporum chrysovirus 1 (EF152346); HvV145S, Helminthosporium victoriae virus 145S (AF297176); MoCV1, Magnaporthe oryzae chrysovirus 1 (NC014462); PcV, Penicillium chrysogenum virus (AF296439).segment 3.

FgV2 dsRNA-3 showed 23% of sequence identity to the L3 capsid protein (CP) of Agaricus bisporus virus 1 (ABV1). According to FgV-ch9 which has high sequences similarity with FgV2, dsRNA3 of might code for the CP (Darissa et al., 2011). The amino acid sequences of dsRNA-4 did not show any significant homology with other known sequences. The deduced amino acid sequence of dsRNA-5 contains a C2H2 zinc finger protein domain at C-terminal region with no homology with revealed sequences except dsRNA-5 product of FgV-ch9 (Darissa et al., 2011).

II. Genome organization of FgV3 and FgV4

In *F. graminearum* strain DK3, three dsRNAs were existed (Fig. 6A). Base on the sequence data analysis, two different dsRNA viruses are infect *F. graminearum* strain DK3, which were referred to as FgV3 and FgV4. FgV3 consists 9,098 nucleotides with 51% of GC content and encoded two large ORFs (Fig. 6B). The two ORFs of FgV3 are separated by 143 nucleotides in length. The 5'- and 3'-UTR were 865 and 44bp long, respectively. No other ORFs of appropriate length were found upstream of position 866. ORF1 of FgV3 was predicted to encode a 1369-aa protein which

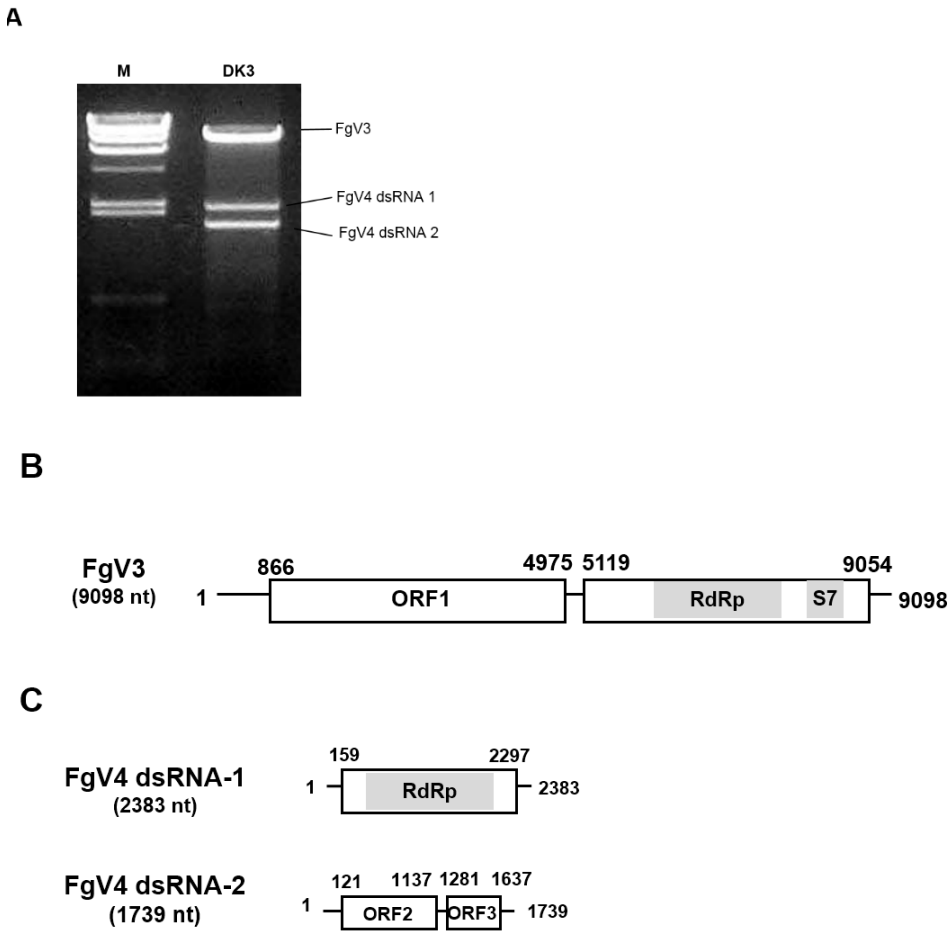

Fig. 6. dsRNA in *F. graminearum* strain DK3. (A) Gel analysis of RNA isolated from dsRNA infected *F. graminearum*. Approximately 500ng of purified dsRNAs were loaded in 1% agarose gel. Lane M contain λ DNA digested with *Hind*III as size markers. Lane DK3 contains purified dsRNAs. (B), (C) Schematic representation of the genome organization of Fusarium graminearum virus 3 (FgV3; B) and Fusarium graminearum virus 4 (FgV4; C) infecting *F. graminearum* strain DK3. Open bars represent open reading frames (ORFs) defined by sequence analysis, and encoding a putative RNA-dependent RNA polymerase (RdRp) and other unknown proteins. Grey bars show conserved RNA-dependent RNA polymerase domain (RdRp) and phytoreovirus S7 protein domain (S7). The non-translated regions are indicated as single line. The nucleotide positions of the initiation and termination codons are indicated above the border of the ORF.

has 44% identity to a hypothetical protein of Grapevine associated totivirus-2. ORF2 encodes 1131-aa protein which has a conserved viral RdRp domain (pfam02123, RdRp_4) and eight conserved motifs of the RdRp in dsRNA mycoviruses (Fig. 7). Furthermore, ORF2 also include phytoreovirus S7 protein domain which has been found also in Penicillium chrysogenum virus (PcV), a *Chrysovirus*.

Genome of FgV4 consists two segments that are referred as dsRNA-1 and dsRNA-2 (Fig. 6C). The nucleotide sequence of dsRNA-1 of FgV4 was 2383 bases long with 56% GC content. The dsRNA-2 has 1739 bp with a GC content of 58%. The 5' and 3' UTRs of the viral RNAs were 158 and 86 bp long in dsRNA-1 and 120 and 102 bp in dsRNA-2. Each 5' and 3' UTRs of dsRNA-1 and dsRNA-2 were presented conserved sequences including 48 and 67 nucleotides 100% identical stretch (Fig. 8). Computer analysis demonstrated that FgV4 dsRNA-1 contains a single putative ORF which has conserved domain of RdRp on its plus-strand RNA. DsRNA-2 encodes two ORFs, however, the minus-strands did not contain any ORF of significant size (Fig. 6B). This single ORF has 38% amino acid sequence identity with the ORF1a of Curvularia thermal tolerance virus (CThTV) which encodes a putative RdRp. In addition, it has 38% identity with the putative RdRp of Cryphonectria parasitica bipartite mycovirus 1 and 26% identity with the

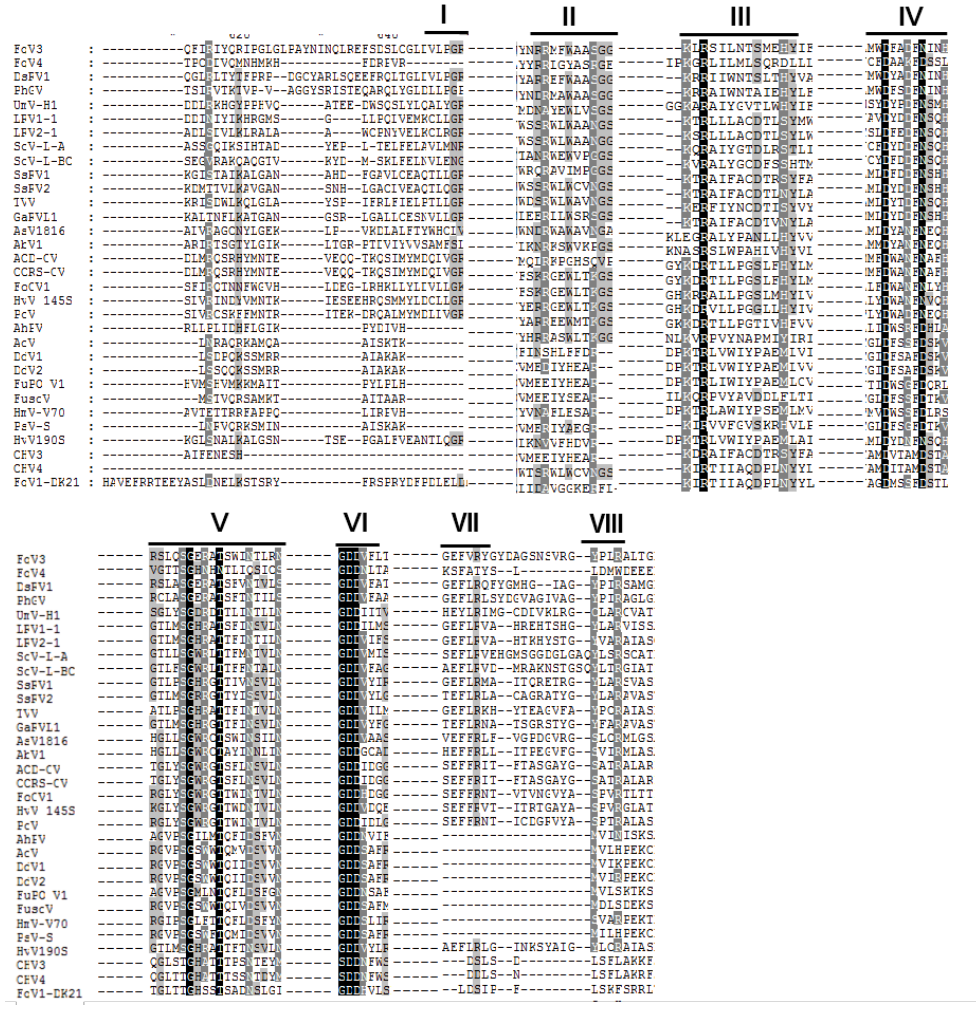

Fig. 7. Amino acid sequence alignment including eight conserved RdRp motifs of FgV3 and FgV4 with those of other dsRNA mycoviruses. Numbers I-VIII indicate the eight conserved motifs in RdRps. The positions of the amino acid sequences are indicated on the right. Abbreviations of virus name and Genbank accession numbers are the following. AbV1, *Agaricus bisporus virus 1* (X94361); ACD-CV, Amasya cherry disease associated chrysovirus (AJ781166); AhPV, *Atkinsonella hypoxylon partitivirus* (NC003470) ; AoV, *Aspergillus ochraceous virus* (ABV30765); AsV1816, Aspergillus mycovirus 1816 (EU289896); CCRS-CV, Cherry chlorotic rusty spot crysovirus (AJ781397) ; CHV3, *Cryphonectria hypovirus 3* (AF188515); CHV4, *Cryphonectria hypovirus 4* (AY307099); DdV1, *Discula destructiva virus 1* (AF316993); DdV2, *Discula destructiva virus 2* (AY033437); DsRV1, Diplodia scrobiculata virus 1 (EF568774); FgV-DK21, Fusarium graminearum virus-DK21 (AY533037); FoCV1, Fusarium oxysporum chrysovirus 1 (EF152346); FuPO V1, Fusarium poae virus 1 (AF047013); FusoV, *Fusarium solani virus 1*

(D55668); GaRV-L1, Gremmeniella abietina RNA virus L1 (AF337175); HmPV-V70, Helicobasidium mompa partitivirus V70 (AB025903) ; HvV145S, *Helminthosporium victoriae virus 145S* (AF297176); HvV190S, *Helminthosporium victoriae virus 190S* (U41345); LRV1-1, *Leishmania RNA virus 1-1* (M92355); LRV2-1, *Leishmania RNA virus 2-1* (U32108); PcV, *Penicillium chrysogenum virus* (AF296439); PhGV, Phlebiopsis gigantea mycovirus (AM111097); PsV-S, *Penicillium stoloniferum virus S* (AM040138); ScV-L-A, *Saccharomyces cerevisiae virus L-A* (U01060); ScV-L-BC, *Saccharomyces cerevisiae virus L-BC* (J04692); SsRV1, Sphaeropsis sapinea RNA virus 1 (AF038665); SsRV2, Sphaeropsis sapinea RNA virus 2 (AF09080); TVV, Trichomonas vaginalis virus (U08999); UmVH1, *Ustilago maydis virus H1* (U01059).

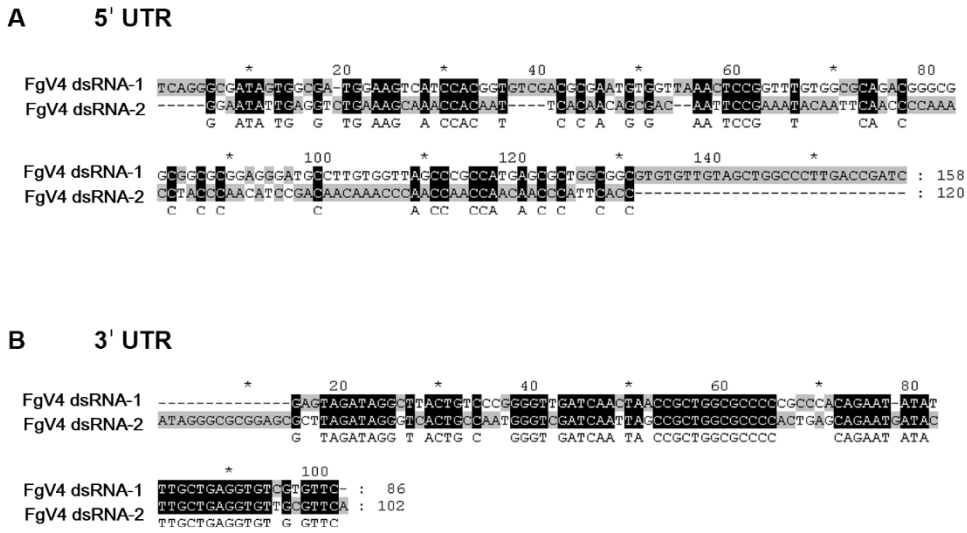

Fig. 8. Conserved sequence elements in FgV2 dsRNA segments. Sequences of the 5′ UTR (A) and 3′ UTR (B) of each dsRNA segment that are identical are reverse highlighted. Black, dark grey, and light grey shading levels represent 100%, 80%, and 60% nucleotides conservation, respectively.

RdRp of a Sugarcane mosaic virus classified in the family of *Potyviridae*. DsRNA2 of FgV4 has two putative ORFs, the deduced amino acid sequence of ORF2 showed amino acid sequence identity to the deduced CThTV 2a protein (30%) and ORF2a of Cryphonectria parasitica bipartite mycovirus 1 (30%). The sequences of FgV3 and FgV4 did not appear to encode a putative coat protein (CP). The complete nucleotide sequences of these dsRNAs were deposited in GenBank under accession numbers GQ140626, GQ140627, and GQ140628 for FgV3, FgV4 dsRNA-1, and FgV4 dsRNA-2, respectively.

III. Phylogenetic analysis

Phylogenetic analysis based on amino acid sequences of RdRp suggested that FgV2-4 were classified in a different mycovirus family. RdRp amino acid sequences of FgV2 is closely related to those of *Chrysoviridae* family including Aspergillus mycovirus 1816 (AsV1816; Hammond et al., 2008) and Magnaporthe oryzae chrysovirus 1 (MoCV1; Urayama et al., 2010). RdRp amino acid sequences of FgV3 classified as a new group within the *Totiviridae* and *Chrysoviridae* families (Fig. 9). On the other hand, those FgV4 were grouped with the family of *Partitiviridae.* The genome organization of FgV2, FgV3, and FgV4 was similar to that of *Chrysoviridae*, *Totiviridae*, and *Partitivirdae* family, respectively. Although they showed

intimate phylogenetic connections to some previously reported dsRNA mycovirus families, FgV3 and FgV4 showed different characteristics since these viruses from same DK3 strain. Together, these phylogenetic analyses demonstrated that these two mycoviruses, FgV2, FgV3 and FgV4, were unrelated to each other and these viruses were also distinct from FgV1 which contained a RdRp phylogenetically related to the *Hypovirdae* family.

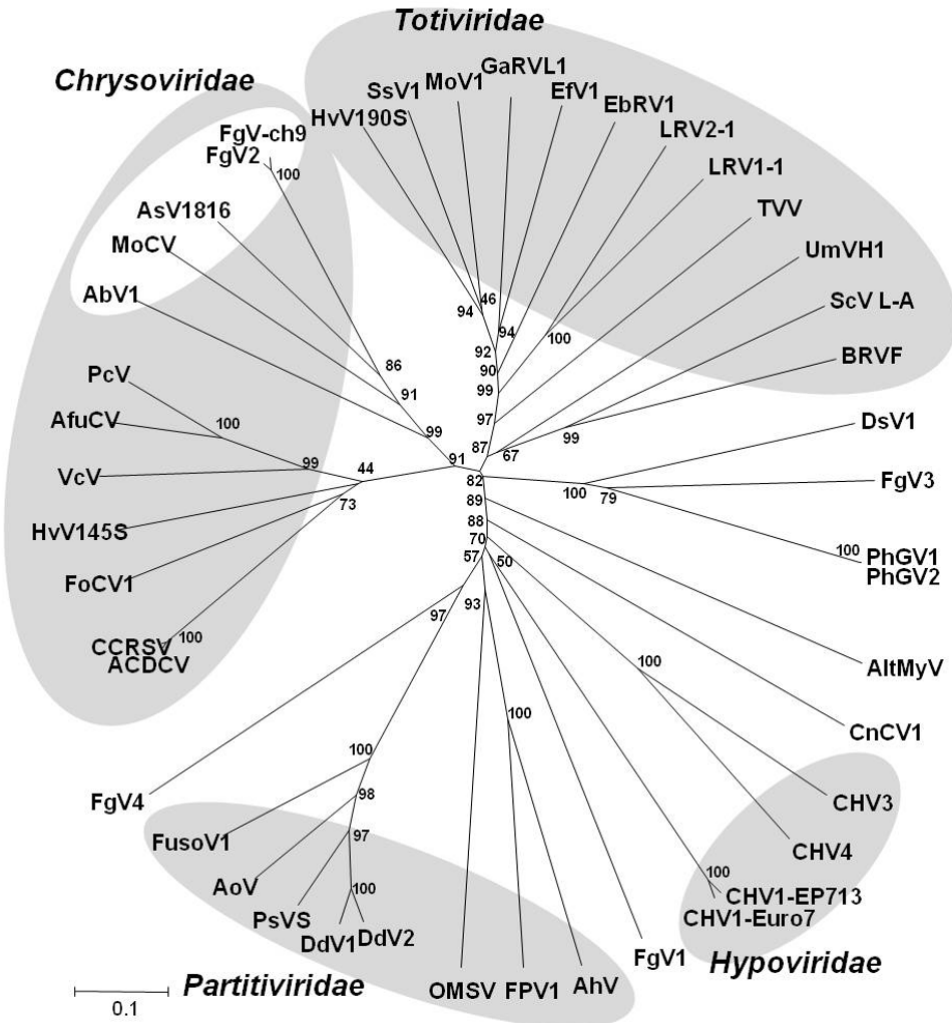

Fig. 9. A phylogenetic tree of FgVs and members of selected mycovirus families based on amino acid sequences of RdRp. Phylogenetic trees were constructed by MEGA version 4.0 program using the neighbor-joining method with 1000 bootstrap replicates. The numbers at each branch display bootstrap values (1000 replicates). Closely related mycoviruses are circled. Abbreviations of virus name and Genbank accession numbers are the following: ABV1, Agaricus bisporus virus 1(X94361); ACDCV, Amasya cherry disease associated chrysovirus (AJ781166); AfuCV, Aspergillus fumigatus chrysovirus (FN178512); AhV, *Atkinsonella hypoxylon virus* (NC003470); AltMyV, Alternaria alternata dsRNA mycovirus (AB368492); AoV, Aspergillus ochraceous virus (DQ270031); AsV1816, Aspergillus mycovirus 1816 (EU289896); BRVF, Black raspberry virus F (EU082131); CCRSV, Cherry chlorotic rusty spot chrysovirus (AJ781397); CHV1-EP713, *Cryphonectria hypovirus1*

EP713 strain (NC001492); CHV1-Euro7, *Cryphonectria hypovirus1* Euro strain (AF082191); CHV3, *Cryphonectria hypovirus* 3 (AF188515); CHV4, *Cryphonectria hypovirus* 4 (AY307099); CnCV1 (GQ290650); Cryphonectria nitschkei chrysovirus 1 bs131 strain; DdV1, *Discula destructiva virus 1* (NC002797); DdV2, *Discula destructiva virus* 2 (NC003710); DsV1, Diplodia scrobiculata virus 1 (EF568774); EbRV1, Eimeria brunetti RNA virus 1 (AF356189); EfV1, *Epichloe festucae virus 1*(AM261427); FgV1, Fusarium graminearum virus 1-DK21 (AY533037); FgV2, Fusarium graminearum virus 2 (HQ343295); FgV-ch9, Fusarium graminearum mycovirus-China 9 (HQ228213); FgV3, Fusarium graminearum virus 3 (GQ140626); FgV4, Fusarium graminearum virus 4 (GQ140627); FoCV1, Fusarium oxysporum chrysovirus 1 (EF152346); FpV1, *Fusarium poae virus* 1 (AF047013); FusoV1, *Fusarium solani virus* 1 (D55668); GaRVL1, *Gremmeniella abietina RNA virus L1* (AF337175); HvV145S, *Helminthosporium victoriae virus 145S* (AF297176); HvV190S, *Helminthosporium victoriae virus 190S* (U41345); LRV1-1, *Leishmania RNA virus 1-1* (M92355); LRV2-1, *Leishmania RNA virus 2-1* (U32108); MoCV1, Magnaporthe oryzae chrysovirus 1 (NC014462); MoV1, Magnaporthe oryzae virus 1 (AB176964); OMSV, Oyster mushroom spherical virus (AY182001); PcV, *Penicillium chrysogenum virus* (AF296439); PhGV1, Phlebiopsis gigantea mycovirus1 (AM111096); PhGV2, Phlebiopsis gigantea mycovirus 2 (AM111097); PsVS, *Penicillium stoloniferum virus S* (AM040138); ScVL-A, *Saccharomyces cerevisiae virus L-A* (U01060); SsV1, *Sphaeropsis sapinea RNA virus 1* (AF038665); TVV, Trichomonas vaginalis virus (U08999); UmVH1, *Ustilago maydis virus H1* (U01059); VcV, Verticillium chrysogenum virus (HM00467).

DISCUSSION

The complete nucleotide sequences of three mycoviruses, isolated from *F. graminearum* strains in Korea, have determined in this study. The genome sequences of FgV1 which causes hypovirulence was analyzed previously. It was confirmed that FgV2-4 were not related to each other and distinct from FgV1. Sequence analysis demonstrated that FgV2 consists five segmented dsRNA and FgV3 and FgV4 are mixed infection. Accumulation of defective RNA and/or satellite dsRNA is a common in mycovirus infection, so it led to confusion in interpreting the organization of viral genome by observing the banding pattern (Gabrial, 1998). Therefore, molecular characterization of the virus is facilitate to clarify whether the crucial genome is segmented or nonsegmented viral RNA with defective or satellite RNAs or whether the complexity is the consequences of mixed infections with more than two viruses (Gabrial, 1998).

Various mycoviruses have been found in same host species, such as *Cryphonectria parasitica*, *Sclerotinia sclerotiorum*, and *Rosellinia necatrix*. In current study, three different viruses which were isolated from *F. graminearum* were characterized based on the genomic organizations. This difference was also related to the different biological features among virus-

infected *F. graminearum* strains. These viruses were transferred to wild-type *F. graminearum* PH-1 by using protoplast fusion for further investigation and it was reported that colony morphology, conidia production, pathogenicity, and trichothecene production showed significant different characteristic among FgVs infected *F. graminearum* PH-1 (Lee et al, 2014). Biological observation demonstrated that FgV1 and FgV2 is associated with hypovirulence traits and FgV3 and FgV4 are symptomless.

The genome sequences of FgV2 has high sequences similarity with the complete genome sequence of FgV-ch9 which is isolated from cereals in China (Darissa et al., 2011). Interestingly, sequence similarity analysis revealed that FgV2 shares entire nucleotide sequence identity of 91%, 97%, 96%, 88% and 85% with FgV-ch9 for dsRNA-1 to -5, respectively. Putative ORF amino acid sequences from the dsRNA-1 to dsRNA-5 showed 98%, 99%, 99%, 94% and 83% of identity with those from the FgV-ch9, respectively. FgV2 and FgV-ch9 were associated with hypovirulence of *F. graminearum*, such as reduced growth rate, abnormal colony morphology, conidiation, and reduced virulence on wheat (Lee et al, 2014; Darissa et al., 2011). This result indicate that FgV2 and FgV-ch9 are closely related each other although they isolated from different region, Korea and China (Yu et al., 2011).

Genome sequencing and phylogenetic analysis suggested the FgV3 and FgV4 were infected same *F. graminearum* DK3 strain and FgV3 was closely related to the *Totiviridae* but FgV4 was related to the *Partitiviridae*. These two viruses infect their host asymptomatically and this phenomenon was identical when they infect host individually (Lee et al, 2014).

FgV3 has conserved phytoreo S7 domain, located at the downstream of RdRp region. The homologs of phytoreo S7 Domain are distributed in non-*phytoreovirus* lineages as well as some unclassified dsRNA mycoviruses including FgV3 (Liu et al, 2012). The conservation of phytoreo S7 domain in many dsRNA viral lineages among different families proposed that this conservation might occurred multiple horizontal gene transfer (HGT) among dsRNA viruses from different families (Liu et al, 2012). The function of this domain in FgV3 infection is not known. It will be of interest to identify the roles of this S7 domain in viral RNA binding or packaging in further investigations.

The amino acid sequences of RdRp of FgV4 has been assigned as an unclassified member of the family *Partitiviridae*. The family *Partitiviridae* consists of fungal and plant viruses with isometric virions with a genome of two dsRNA segments each of 1.4-2.2 kb in general. Also, the viruses are associated with latent infections in fungal or plant hosts (Ghabrial and Suzuki,

2009). In genome organization and phylogenetic analysis, FgV4 was related to *Partitiviridae* family but separate from other *Partitivirus*. It is possible that FgV4 might build new subgroup in the family of *Partitiviridae.*

In the present study, the complete genome sequences of three Fusarium graminearum virus, FgV2, FgV3, and FgV4, have analyzed. The result showed that FgV2, FgV3, and FgV4 are belong to the family of *Chrysoviridae*, *Totiviridae*, *Partitiviridae*, respectively and form the new phylogenetic cluster within the virus family. Whole genome sequence information will be necessary to understand pathogenic determinant of FgVs functions at amino acid level. Our study, together with previous studies (Chu et al, 2004; Kwon et al, 2007; Lee et al, 2014), shows that its association with hypovirulence of its fungal host, might constitute a field study for further investigations that would facilitate the virus or its genes for the biological agent of *F. graminearum*.

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CHAPTER II

Characterization of the *FgHal2* gene required for host defense against Fusaruim graminearum virus-1 infection

ABSTRACT

The Fusarium graminearum virus 1 (FgV1) infect *Fusarium graminearum* and it has hypovirulence traits such as reduced mycelial growth, increase pigmentation, and reduced pathogenicity in host. In previous study, it confirmed that many *F. graminearum* genes are differentially expressed upon FgV1 infection. Several of these genes might be involved in the persistence and maintenance of the virus life cycle. One of the host gene, *FgHal2*, is bifunctional gene which has a conserved 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) domain or inositol monophosphatase (IMPase) domain, shows reduced expression in response to FgV1 infection. Targeted gene deletion and over-expression mutants were generated to verify the possible role(s) of *FgHal2* and its interaction with FgV1. The phenotype of *FgHal2* deletion mutant (Δ*FgHal2*) showed retarded growth, defect in aerial mycelia formation, and reduced pigmentation whereas over-expression mutant (*FGHAL2* OE) had normal morphological phenotype compared to the wild type (WT). The conidia production of Δ*FgHal2* was reduced significantly and abnormal conidia was observed frequently. The *FgHal2* expression level was decreased by FgV1 infection at 120 h post-infection (hpi) whereas the levels were 9-fold greater for both the virus-free and virus-

infected *FGHAL2* OE than for the WT. Comparing to the wild-type, FgV1 RNA accumulation was not increased in the Δ*FgHal2*-VI from 48 to120 hpi. However, FgV1 RNA accumulation in the *FGHAL2* OE-VI was reduced at 120 hpi relative to the WT but was similar to that of the WT at 72 hpi. The vertical transmission rate of FgV1 in the Δ*FgHal2* was dramatically decreased, suggesting that *FgHal2* might be required for maintenance of FgV1 in host cell. Together, these results indicate that the putative 3'(2'), 5'-bisphosphate nucleotidase gene, *FgHal2*, has diverse biological functions and might facilitate the maintenance of FgV1 in the host fungus.

INTRODUCTION

Mycoviruses are widely distributed in fungi including plant-pathogenic fungi, mushroom, and medical fungi (Xie and Jiang, 2014). Mycoviruses have genomes of dsRNA, ssRNA, or DNA and classified in several families, including *Totiviridae, Chrysoviridae, Partitiviridae*, *Hypoviridae,* and *Narnaviridae*. While many mycoviruses infect their fungal hosts latently, some viruses are associated with hypovirulence, i.e., they reduce the virulence of the pathogenic fungus, and affect growth and sexual and asexual reproduction (Pearson et al., 2009). From an agricultural perspective, hypovirulence-asscociated mycoviruses are considered as biological control agent for plant pathogenic fungi. (Kim et al., 2013; Ghabrial and Suzuki, 2009). In addition, the study of the mycovirus is important sources to understand fungal biology, host-microbe interaction, and evolution and ecology of viruses.

Most viruses have relatively small genomes that encode few genes, and therefore viruses depend almost on host cellular machinery for replicating their genomes. A wide variety of host cellular proteins or viral elements are reported to play important roles in the intracellular multiplication of plant or animal viruses (Whitman and Wang, 2004). However, host factor that related

to life cycles of mycoviruses are largely unknown. Also, fungal host proteins which modulate host gene expression and related to the defenses responses against viral infection are not revealed yet. Therefore, it will be interest to characterize viral determinants and to identify the fungal host genes involved in the mycovirus-host interaction (Ghabrial and Suzuki, 2009).

Previous research demonstrated that broad range of host genes expression were affected by mycovirus infections and these changes led to hypovirulence or phenotypic alterations in the host (McBride et al., 2013; Lee et al., 2014; Allen and Nuss, 2004). Several host genes in *Cryphonectria parasitica* have been identified that regulated by Cryphonectria hypovirus 1 strain EP713 (CHV1). Theses identified genes were related to transmission or inheritance of CHV1 and also required for female fertility, conidiation, virulence, fungal stress response (Sun et al., 2009; Gao et al., 2013; Baek et al., 2014).

Fusarium graminearum is a homothallic ascomycetes and the major causal organism of Fusarium head blight (FHB) and ear rot (Desjardins and Proctor, 2007). Fusarium head blight (FHB) is a destructive disease of wheat, barley, and other small grains that has caused reduction in grain yield and quality. (McMullen et al., 1997). In addition, this plant-pathogenic fungus produces mycotoxins such as trichothecenes and zearalenone in cereals, which is known to cause food refusal, vomiting, and depressed immune

function (Omurtag, 2008). In previous study, several mycoviruses have been isolated and identified in Fusarium species including *Fusarium graminearum* (Kim et al., 2013). Among the Fusarium graminearum viruses, Fusarium graminearum virus 1 strain DK21 (currently named FgV1) has been reported to alter ability of virulence of plant pathogenic fungus. The FgV1-infected fungus showed reduced virulence, a reduced mycelial growth rate, increased pigmentation, and inhibition of the mycotoxin production (Chu et al., 2002). FgV1 has about 6.6 kb in length of RNA genome which encodes four ORFs (Kwon et al., 2009b). FgV1 generally presents as double-stranded RNA forms in host cell but expression strategy of its genome is similar to that of potex-like viruses and is phylogenetically similar to members of the genus *Hypovirus* based on the alignment of RdRp domains (Kwon et al., 2009b).

In previous researches, it was observed that diverse fungal host genes are differentially expressed by FgV1 infection base upon transcriptomic and proteomic approaches. These host genes are related to the cell signaling pathway, stress response, post-transcriptional gene silencing, differentiation, and other processes (Kwon et al., 2009a; Cho et al., 2012; Lee et al., 2014). We have speculated that some of those genes might be involved in maintaining the life cycle of FgV1 as host factors (Kwon et al., 2009a; Son et al., 2013). For example, *Hex1*, a component of the Woronin body, is

important to the accumulation of FgV1 viral RNA and maintain the cellular integrity and pathogenicity of *F. graminearum* (Son et al., 2013). Unfortunately, the molecular mechanisms underlying Hex1 proteins-FgV1 interactions are not clearly understood (Son et al., 2013) and other host genes in *F. graminearum* that may directly and/or indirectly affect the life cycle of FgVs have not been identified.

The current study present the one of *F. graminearum* genes, *FgHal2*, which shows reduced gene expression level (at the level of transcription and translation) in response to FgV1 infection (Kwon et al., 2009a; Lee et al., 2014). *FgHal2* is an ortholog of the yeast gene *Hal2* (*MET22*) and contain conserved domain of 3'-phosphoadenosine-5'-phosphate (pAp) phosphatase, also known as 3'(2'), 5'-bisphosphate nucleotidase. Met22p removes the 3' phosphate from pAp, thus producing AMP, and also hydrolyzes 3'-phosphoadenosine 5'-phosphosulfate (pApS) in methionine biosynthesis in the sulfate assimilation pathway (Hudson and York, 2012). Met22p can suppress viral RNA recombination of Tomato bushy stunt virus (TBSV) in yeast and is related to Xrn1p 5'-3' ribonuclease, a known suppressor of viral RNA recombination (Jaag and Nagy, 2010). SAL1, one of the *Arabidopsis* orthologs of yeast *Hal2*, is bifunctional enzyme that catabolizes inositol 1, 4, 5-trisphosphate (IP3) and pAp (Hudson and York, 2012; Estavillo et al.,

2011). It also hinders post-transcriptional gene silencing by degrading the exoribonuclease inhibitor pAp, and functions in stress-responses and developmental processes in plants (Gy et al., 2007; Estavillo et al., 2011).

In this study, the evidences were provided that *FgHal2* gene in *F. graminearum* is down-regulated following FgV1 infection. To investigate the role(s) of *FgHal2* in *F. graminearum*, gene deletion and gene over-expression mutants were used in this study. The deletion of *FgHal2* affect reduced conidiation, mycelial growth, and the production of secondary metabolites in *F. graminearum*. Furthermore, FgV1 viral RNA accumulation and vertical transmission of FgV1 via conidia were decreased in deletion of *FgHal2*. Together, these results indicate that *F. graminearum* modulate the expression of its major multi-function genes, *FgHal2*, as an antiviral host defense response.

MATERIALS AND METHODS

I. Fungal strains and media

The PH-1 strain of *F. graminearum* was used as the WT in this study (Lee et al., 2014). The gene deletion and over-expression mutants were generated from the WT, and FgV1 was transferred to each strain by hyphal anastomosis as described previously (Lee et al., 2014). All strains used in this study are listed in Table 1. For extractions of total RNA and genomic DNA, all strains were grown in 50 ml of liquid CM (Leslie and Summerell, 2006) at 25°C for 5 days on a rotary shaker at 150 rpm. The WT and mutant strains were stored in 15% (v/v) glycerol at -80°C and were reactivated on CM agar medium.

II. Sequence analysis

Nucleotide sequence and amino acid sequences were obtained from the NCBI database and Fusarium comparative database (Fusarium Comparative Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org). Amino acid sequences alignment used in this study were obtained using the MegAlign program (DNASTAR, Inc. USA) and GeneDoc programs (http://www.nrbsc.org/gfx/genedoc/). A conserved domain search and sequence similarity analysis with other organisms were

Table 1. *Fusarium graminearum* strains used in this study

Strain	Description	References
WT-VF	Wild-type *F. graminearum* PH-1 (lineage 7)	Lee *et al*. (2014)
WT-VI	Wild-type PH-1 infected with FgV1	Lee *et al*. (2014)
Δ*FgHal2*-VF	*FgHal2* gene deletion mutant in WT-VF genetic background	This study
Δ*FgHal2*-VI	FgV1 introduced into Δ*FgHal2*-VF by hyphal anastomosis	This study
FGHAL2 OE-VF	*FgHal2* gene over-expression mutant in WT-VF genetic background	This study
FGHAL2 OE-VI	FgV1 introduced into *FGHAL2* OE-VF by hyphal anastomosis	This study
*FgHal2*C-VF	*FgHal2* gene complement mutant in WT-VF genetic background	This study
*FgHal2*C-VI	FgV1 introduced into *FgHal2*C-VF by hyphal anastomosis	This study

conducted using the NCBI BLAST program.

III. Construction of *FgHAL2* gene deletion, over-expression, complementation mutants

DNA constructs for targeted gene deletion, complementation, and over-expression were amplified by the double-joint (DJ) PCR method as described previously with slight modification (Son et al., 2013). Briefly, the 5′- and 3′-flanking regions of the FgHal2 gene were amplified from *F. graminearum* PH-1 and geneticin-resistance gene cassette (gen) as a fungal selectable marker amplified from vector pII99 using primers GenF and GenR. Amplified DNA were fused by DJ PCR under the PCR conditions previously described (Yu et al, 2004). Primer sets used in this study are listed in Table 2. To generate complemented strain of the *FgHal2* gene deletion mutant, the DNA fragment carrying the native promoter located in upstream of *FgHal2* and *FgHal2* ORF was amplified with 9532 5F2/9532 5′ RC primers and then fused with the green fluorescent protein gene (gfp) and the hygromycin (hyg)-resistance cassette, which amplified from the pIGPAPA vector (Lin et al., 2011) by DJ PCR. For generating *FgHal2* overexpression mutants, the 5′-end and 3′-end of the *FgHal2* region were amplified by primer sets 9532 5F/9532 5F Gen-R primer and EF1 9532-3F/9532 OE-3R, respectively. The gen-$P_{ef1\alpha}$

construct, including the gen-resistant cassette and the elongation factor 1α promoter ($P_{ef1\alpha}$) from *F. verticillioides*, was amplified from pSKGEN (Lee et al., 2011b) with Gen_EF1 F/EF1 pro R primers. Amplicons were joined in the same way as described above, and a final PCR product was generated using the nested primers. All final DNA constructs were applied for fungal protoplast transformation. Fungal protoplasts were treated with PEG for transformation as previously (Son et al., 2013). Transformants were selected on potato dextrose agar (PDA) supplemented with 50 μg/ml of hyg or gen. All transgenic strains were confirmed by Southern blot (described below), FgV1 was introduced into the virus-free transformant strains through hyphal anastomosis. Viral infection was confirmed by RT-PCR using FgV1-specific primer pairs. All primers that used for mutant generation were listed in Table 2.

IV. **DNA extraction and Southern blot hybridization**

After fungal strains were incubated for 5 days on CM broth, mycelia were collected by filtration through Whatman 3MM filter paper (GE Healthcare, Sweden), washed with distilled water, pressed onto paper towels to remove the excess water, and stored at -80°C. Genomic DNA extraction protocol was same method with previously described (Lee et al., 2011). For

Table 2. Primer sets for generation of mutant in this study.

Primer	Sequence (5'→3')	Description
9532 5F	GGTGACAGTTTTCTCCGACAT	5' flanking region of FgHal2 with a tail for geneticin gene cassette fusion
9532 5R	CCTTCAATATCATCTTCTGTCG**GGTTGCTAACGGTCGATTGA**	
9532 3F	GCACAGGTACACTTGTTTAGAGAACCGTTAGGTGGAAGAAAG	3' flanking region of FgHal2 with a tail for geneticin gene cassette fusion
9532 3R	GAATGAGCAGATGAGGTGAAGT	
9532 nest F	CGCTCCTCCTCGTCCATAC	nest primers for third fusion PCR for amplification of FgHal2 deletion construct
9532 nest R	GACAGCCATATCGACACCAAT	
Gen F	CTCTAAACAAGTGTACCTGTG	Forward and reverse primers for amplification geneticin cassette from the pII99 vector
Gen R	CGACAGAAGATGATATTGAAG	
Hyg+GFP F	GTGAGCAAGGGCGAGGAGCTG	GFP and the hygromycin resistance cassette (hyg) from the pIGPAPA vector (ref)
Hyg+GFP R	GGCTTGGCTGGAGCTAGTGGAGG	
9532 5F2	GCCTTGGTGTTTCATCGCATACG	5' flanking region of FgHal2 for complementation construct
9532 5' RC	TGACTGCTTGGCGCTCAGGAC	
9532 HygR+GFP F	GTCCTGAGCGCCAAGCAGTCA**GTGAGCAAGGGCGAGGAGCTG**	for amplification GFP-hyg cassette from the pIGPAPA vector (ref) with a tail for FgHal2 5' or 3' flanking region
HygR+GFP-R 9532 3F	CCTTTCTTCCACCTAACGGTT**GGCTTGGCTGGAGCTAGTGG**	
9532 3'FC	AACCGTTAGGTGGAAGAAAGG	3' flanking region of FgHal2 complementation construct
9532 3'f RC	CTCAAACCTGGCAGTGCACGAC	
Gen_EF1 F	GGGGCGTCGGTTTCCACTATC	gen-P_{efla} from the pSKGen vector (ref)
EF1 pro R	CTTTGAAGATTGGGTTCCTTTTGTGATA	
9532 5F Gen-R	GATAGTGGAAACCGACGCCCC**GGTTGCTAACGGTCGATTGA**	5' flanking region of FgHal2 with a tail for ef1a promoter replacement
EF1 9532-3F	TATCACAAAAGGAACCCAATCTTCAAAG**ATGGCCGCCCCATCTTACG**	3' flanking region of FgHal2 with a tail for ef1a promoter replacement
9532 OE-3R	CGTTACACTTCCGGGCTTCTTG	

the Southern blot hybridization of WT and transgenic mutants, 10 μg of genomic DNA was digested with the appropriate enzyme. Loaded on 0.8 % Agarose gel and after gel electrophoresis, capillary blotting, radiolabeling of DNA probes and hybridization were followed by described protocols (Lee et al., 2011). Hybridized blots were exposed to phosphoimaging screens (BAS-IP MS 2040, Fuji Photo Film, Japan) and visualized using a BAS-2500 image analysis system (Fuji Photo Film Co, Japan).

V. Mycelial growth and conidiation test

The morphologies of the virus-free and virus-infected WT and transformed mutant strains were observed at 5 dpi on PDA, CM, and minimal medium (MM; 0.05%, KCl, 0.2% $NaNO_3$, 3% sucrose, 1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.02% trace element, 2% agar) at 25°C. The radial growth was measured on PDA after 3, 4, and 5 days at 25°C. The experiment was repeated three times independently. To assess colony surface hydrophobicity, a 10 μl droplet of 2.5 % of bromophenol blue solution was applied to the top of 4-day-old fungal colonies grown on CM agar; the colonies were photographed 10 min later.

For observation of conidial production, the virus-free and FgV1-infected strains were incubated for 3 days on CM liquid media at 25°C.

Mycelia were collected by filtration through Whatman 3MM filter paper and washed twice with distilled water. The harvested mycelia were spread on yeast malt agar (YMA) and incubated for about 48 h at 25°C under near UV light (wavelength: 365 nm, HKiv Import and Export Co., Ltd., Xiamen, China). Conidia were collected with distilled water, filtered through cheese cloth, and suspended in distilled water. A 10-μl volume of this suspension (1×10^5 conidia/ml) was placed in 5 ml of carboxymethyl cellulose (CMC) medium (1.5% carboxymethyl cellulose, 0.1% yeast extract, 0.05% $MgSO_4H_2O$, 0.1%NH_4NO_3, and 0.1% KH_2PO_4) for 120 h at 25°C on a rotary shaker (150 rpm). The conidia produced in the CMC culture were filtered through six layers of sterilized gauze, collected by centrifugation, and counted using a hemocytometer. The experiment was independently repeated three times.

VI. Microscopic observation of conidial and hyphal morphology

To assess conidial morphology and length, the conidia produced by each strain on YMA or CMC were collected and observed with differential interference contrast (DIC) optics using a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany). The localization of GFP in the complementation strain was determined with the DE/Axio imager A1

microscope and a GFP filter (470/40 nm excitation, 495 nm dichroic, 525/50 nm emission).

VII. Virulence assay on flowering wheat heads

A virulence test was performed on wheat cv. Jokyoung as described previously (Son et al., 2013). Briefly, mycelial plugs were incubated in CMC liquid culture for 5 days to prepare the conidial inoculum. The cultures were filtered through six layers of sterilized gauze and washed with distilled water; conidia in the filtrate were collected by centrifugation. A 10-μl volume of the conidial suspension (1×10^5 conidia/ml) of each strain was injected into 10 replicate wheat head florets at early–mid anthesis. Virulence was observed 14 days after inoculation by quantifying the percentage of head blight symptom on spikelets. Statistical analysis was conducted with the PASW statistics software 20.0 (IBM SPSS Inc., Armonk, U.S.A.).

VIII. Total RNA extraction and gene expression level analysis

Total RNAs of the virus-free or virus-infected WT and mutant strains were extracted from the mycelia incubated on CM liquid for 48, 72, and 120 h. Total RNA extraction and cDNA synthesize was performed as previously described (Lee et al., 2014). qRT-PCR was conducted using CFX96 Real-

Time PCR System (Bio-Rad, USA) with the SsoFast™ EvaGreen® Supermix (Bio-Rad, USA) according to the manufacturer's instructions. After initial denaturation at 95°C for 5 min, 40 cycles consisted of 5 s at 95°C and 5 s at 58°C. Two endogenous reference genes, ubiquitin C-terminal hydrolase (UBH, locus FGSG_01231; (Kim, 2011)) and elongation factor 1α (EF1α, locus FGSG_08811), were used as internal controls to normalize qRT-PCR results. All primers for qRT-PCR were listed in Table 3.

IX. Vertical transmission

Vertical transmission of FgV1 by virus-infected WT and mutant strains was measured based on conidial sporulation and subculturing on PDA for three generations. Conidia were harvested from FgV1-infected strains as described above, and 10 to 15 spores of this first generation were spread onto one PDA plate incubated for 2 to 3 days for spore germination. At least 50 conidia of each strains were spread onto PDA plates per generation. Germinated single spores were transferred to new PDA plates, each containing 30 independent germinates. These were incubated at 25°C for an additional 5 days. The development of morphology and pigmentation of the colonies were assessed first, and each colony was then confirmed the presence of FgV1 RNA by using RT-PCR with virus-specific primers (Takara Bio Inc. Japan). The FgV1

infected colonies were then used to produce a second generation of conidia, and the process was repeated to generate and assess a third generation. The percentage of conidia that produced colonies containing FgV1 RNA was determined for each generation.

Table 3. Primer sets for qRT-PCR in this study.

Primer	Sequence (5'→3')	Description
9532 RT-F	GCATTACCCAGGACAGTGTTCG	For real-time PCR of FgHal2
9532 RT-R	GAATGTCAGTGACCTCGCCACC	
1708 RT-F	CATGGGCCTGGGTAACACGACC	For real-time PCR of fg01708
1708 RT-R	GCTCATCTTTCTCCCAGCCGTC	
7103 RT-F	GCAGTTCGCGGACAAGGCACA	For real-time PCR of fg07103
7103 RT-R	ATGAGTCGGCAAGCTTGGTAATG	
EF1 RT-F	CAAGGCCGTCGAGAAGTCCAC	For real-time PCR of EF1a as internal control
EF1 RT-R	TGCCAACATGATCATTTCGTCGTA	
UBH RT-F	GTTCTCGAGGCCAGCAAAAAGTCA	For real-time PCR of UBH as internal control
UBH RT-R	CGAATCGCCGTTAGGGGTGTCTG	
FgV1 RT-F	CAGCATTTATTTGACCGCCACTCC	For real-time PCR of FgV1 3638-3818nt region
FgV1 RT-R	CCCGTAAGCAGTCGAGCACCAGAG	
AurJ-F	AAAAAGCAGCCAAGGAGCAT	For detect AurJ (fg02326) gene
AurJ-R	TTCTGATGACACGCTCCCGTA	
Os2-F	TTGTCAAGTCGCTACCCAAG	For detect os-2 (fg09612) gene
Os2-R	ATGTATCAACAGGCAGATCGG	
Pks12-F	AATGGCTTCTTGCACATTTCC	For detect pks12(fg02324) gene
Pks12-R	GCAATCCGATCCATGAACAA	
Gip2 RT-F	CACCAGCCCTACACCATCTAA	For detect Gip2 (fg02320)
Gip2 RT-R	TTTCCAAAGCGAGAAACAGC	
06799 RT-F	GGCTATGCGAACGTTGGGGTCAC	For detect Xrn1 (fg06799) gene
06799 RT-R	TTCGGTAAACCAAGGTGCTGTGC	
10716 RT-F	CCAAGCATCGCAAACGCACAAG	For detect Xrn1 (fg10716) gene
10716 RT-R	CGCGTCATGCCAGGTCCGTTGTA	

RESULTS

I. Sequence analysis of *FgHAL2*

The genomic sequence of *FGSG_09532*, corresponding to the *FgHal2* gene, was determined at the Munich Information Centre of Protein Sequences (MIPS) and the Fusarium comparative database (Fusarium Comparative Sequencing Project, Broad Institute of Harvard and MIT, (http://www.broadinstitute.org). *FgHal2* is 1074 bp in length and encodes 357 amino acids which contain 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) or the inositol monophosphatase (IMPase) superfamily domain. Also, it was predicted that *FgHal2* included the substrate binding-site and the putative Li^+/Na^+ binding sites (Fig 1). The BLASTp search of *FgHal2* against other organism including yeast, Arabidopsis, and filamentous fungi indicate that 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) or the inositol monophosphatase (IMPase) superfamily domain is relatively conserved among all orthologs and *FgHal2*. Additionally, amino acid sequences of *FgHal2* and *FgHal2* orthologs were aligned using the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/). The *FgHal2* in *F. graminearum* shared a homolog at the amino acid sequence with *Saccharomyces cerevisiae* Met22 protein (45%). *FgHal2* also showed high sequence identity with *F. oxysporum*

(95%), *F. verticillioides* (95%), *Neurospora crassa* (76%), and *Magnaporthe oryzae* (78%).

Sequence analysis in the promoter region of the *FgHal2* gene revealed the presence of two stress response element motifs (STRE), 5′-CCCCT-3′ and 5′-AGGGG-3′, which located in the promoters of many stress-related genes (Martinez-Pastor et al. 1996). Stress-response genes are bind to these stress response elements in response to mild heat shock, starvation, osmotic stress, alcohol, and weak acids (Martinez-Pastor et al. 1996).

The *F. graminearum* chromosome has other two putative genes that contain a PAP phosphatase-like domain, locus at the *Fgsg_07103* and *Fgsg_01708*. The comparison of the deduced amino acid sequence of *FgHal2* with those of two hypothetical proteins showed 42% and 35% sequence identities, respectively (Fig. 2A). When quantitative real-time RT-PCR (qRT-PCR) were carried out to quantify the alteration of expression level of *Fgsg_07103* and *Fgsg_01708* after FgV1 infection, only the *FgHal2* gene was down-regulated after virus infection; transcription levels of *Fg07103* and *Fg01708* genes did not show significantly change (Fig. 2B). This result

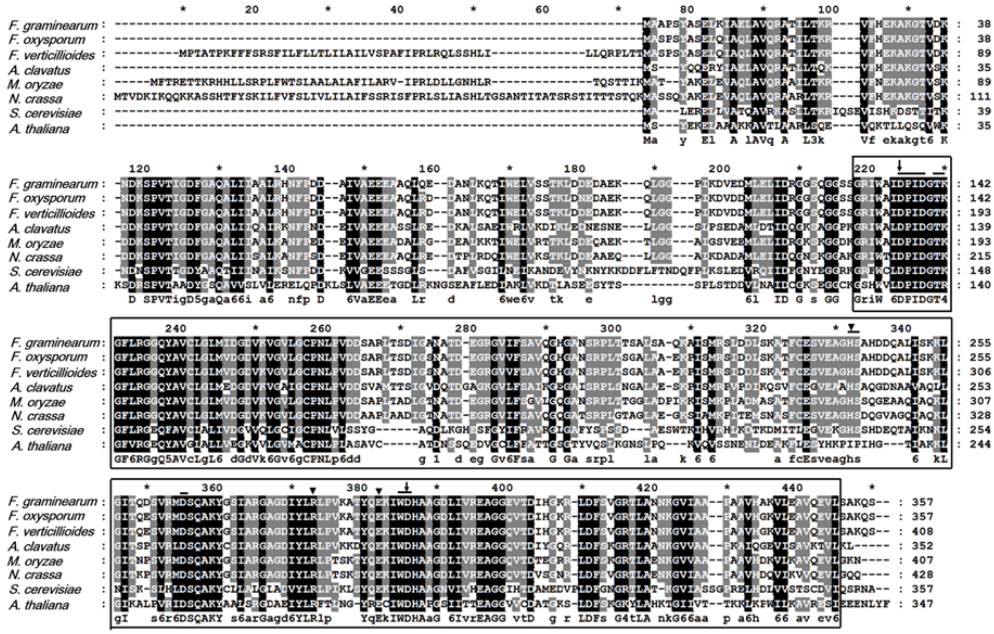

Fig. 1. Amino acid sequence alignment of FgHal2 corresponding to the conserved PAP phosphatase-like domain of the predicted FgHal2 protein and orthologs. The positions of the amino acid sequences are indicated on the right. Species and GenBank accession numbers are the following: *Arabidopsis thaliana* (NP201205.1); *Aspergillus clavatus* (XP001268064.1); *Magnaporthe oryzae* (XP003719499.1); *Fusarium graminearum* (XP389708.1); *Fusarium oxysporum* (EGU82355.1); *Fusarium verticillioides* (EWG42162); *Neurospora cassa* (EAA28448.2); *Saccharomyces cerevisiae* (NP014577.1) The conserved amino acids are shaded. The alignment was generated with GeneDoc software. The PAP phosphatase-like domain [cd01517] is boxed; the conserved active site residues are indicated by a bar above the sequence; the substrate binding-site residues are indicated by arrowheads; and the putative Li^+/Na^+ binding sites are indicated by black arrows.

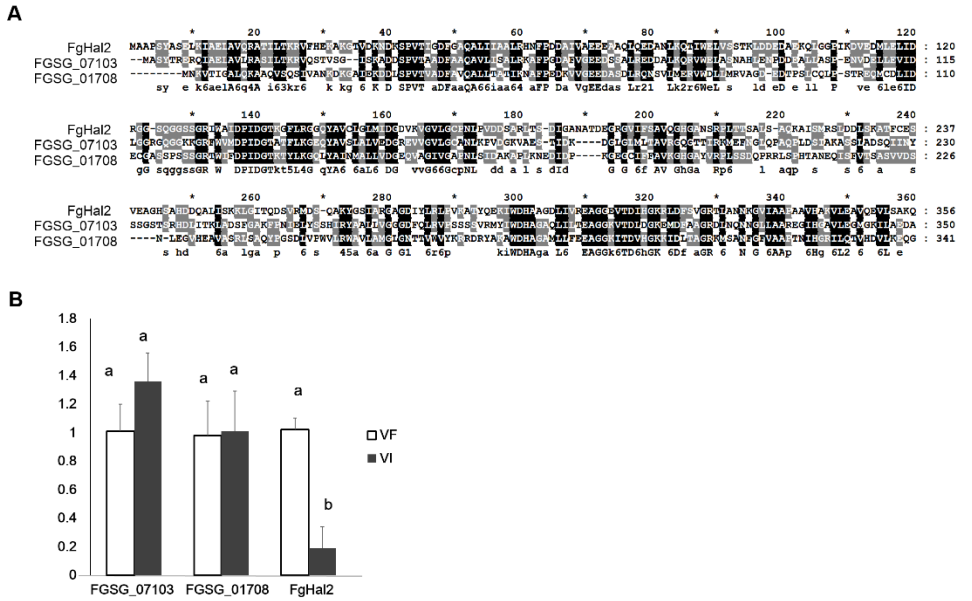

Fig. 2. Comparison of genes encode the PAP phosphatase-like domain in *F. graminearaum* (A) Amino acid sequence alignment of the PAP phosphatase-like domain in *F. graminearaum.* The predicted FgHal2 protein, hypothetical protein FGSG_07103, and FGSG_01708 were used for this analysis. The positions of the amino acid sequences are indicated on the right. Protein names and GenBank accession numbers are as follows: *Fusarium graminearum* FGSG_09532 (XP389708.1); *Fusarium graminearum* FGSG_07103 (XP387279.1); *Fusarium graminearum* FGSG_01708 (XP381884.1) (B) Relative mRNA expression in the virus-free and virus-infected strain *F. graminearum* PH-1 WT strain. Relative transcript levels of *fg07103*, *fg01708*, and *FgHal2* were normalized to elongation factor 1 and ubiquitin C-terminal hydrolase. cDNAs were generated from total RNA extracts obtained after 120 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on the Tukey test. VF, virus-free; VI, virus-infected.

suggest that expression of *FgHal2* might be associated with FgV1 infection.

II. Generation of mutants

To investigate the role of *FgHal2* in *F. graminearum*, gene deletion and gene over-expression mutants were generated (Fig. 3). For construction of the gene deletion mutant, the *FgHal2*-upstream and -downstream regions were amplified from wild-type (WT) *F. graminearum* PH-1 genomic DNA by PCR and fused to the geneticin resistance cassette. The fused DNA construct was transformed by protoplast-mediated fungal transformation, and *FgHal2* gene was replaced with *gen* by homologous recombination. For construction of the complementation strain of *FgHal2*, the ORF of *FgHal2* and the GFP fusion construct under the control of the *FgHal2* native promoter and hygromycin cassette were inserted into the genome of the *FgHal2* deletion mutant. To generate an *FgHal2* over-expression strain, the EF1α promoter of *F. verticillioides* was added to upstream of *FgHal2* ORF. All transformed fungal mutants were confirmed by southern blot analysis (Fig. 4). qRT-PCR of the virus-free strains confirmed that the transcript level of *FgHal2* was greatly reduced in the deletion mutant, greatly increased in the over-expression mutant, and observed similar level of expression in the WT and the

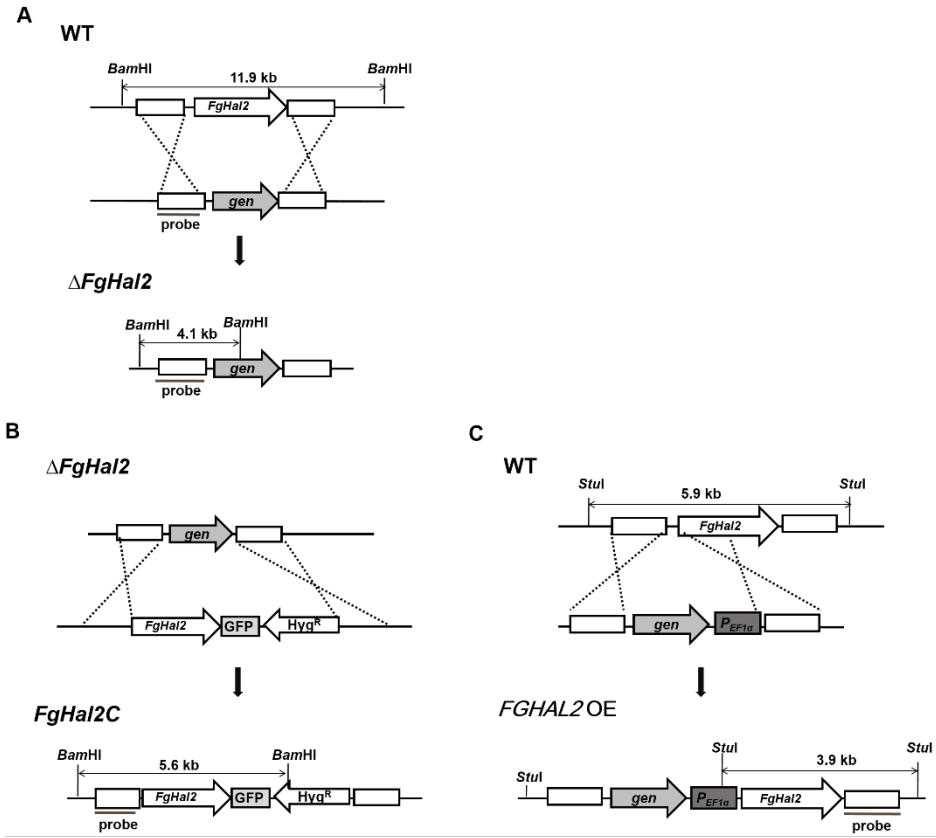

Fig. 3. Generation of *FgHal2* gene deletion, complementation, and overexpression mutants of *F. graminearum*. (A) Schematic representation of the homologous gene recombination strategy used to generate the *FgHal2* deletion mutants (left), *FgHal2* complementation mutant (middle), and *FgHal2* overexpression mutant (right). The complementary of the *FgHal2* mutant strain was fused with GFP. The promoter was replaced with the *EF1α* promoter in the *FGHAL2* OE strain. WT, *F. graminearum* wild-type strain PH-1; Δ*FgHal2*, *FgHal2* gene deletion mutant; *FgHal2C* complementary of the *FgHal2* and GFP-fused mutant; *FGHAL2* OE, *FgHal2* overexpression mutant.

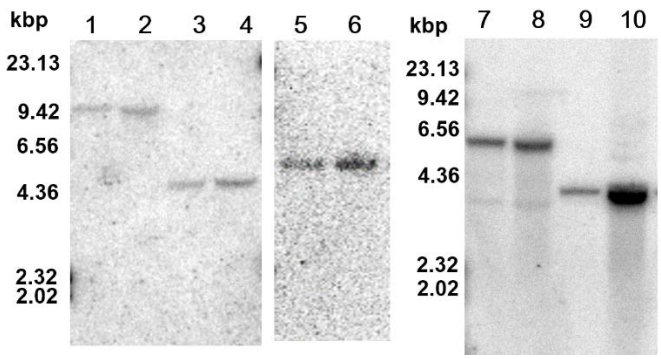

Fig. 4. Southern blot hybridization of *F. graminearum* mutant strains. Sizes of the DNA standards are indicated to the left of the blot. Lane 1-6 indicate Southern blot images of *Bam*HI-digested genomic DNA of each strain. Lane 1 and 2 represent the WT, lane 3 and 4 represent Δ*FgHal2*, and lane 5 and 6 represent the complementary strain (*FgHal2C*). ^{32}P-labeled DNA fragment of the 5' flanking region of the *FgHal2* gene was used as a probe for southern blot for hybridization (Lane 1-6). In Lane 7-10 indicate Southern blot images of *Stu*I-digested genomic DNA of each strain. Lane 7 and 8 represent the WT, and lane 9 and 10 represent *FGHAL2* OE. ^{32}P-labeled DNA fragment of the 3' flanking region of the *FgHal2* gene was used as a probe (Lane 7-10). In lane 1–10, odd-numbered lanes indicate virus-free strains and even-numbered lanes indicate virus-infected strains.

complementation mutant (Fig. 5). All fungal strains used in this study are listed (Table 1).

III. Impact of FgV1 infection on *FgHal2* gene expression level

To examine whether the expression level of *FgHal2* mRNA differed among the *F. graminearum* strains, FgV1 was introduced into the virus-free *FgHal2* gene deletion strain, complementation strain, and over-expression strain through hyphal anastomosis. As indicated by qRT-PCR, the expression level of *FgHal2* mRNAs was decreased approximately 5-fold in the FgV1-infected WT compared to that of the virus-free WT at 120 h post-inoculation (hpi) (Fig. 5). The *FgHal2* transcript level was also significantly lower in the virus-infected WT and the complemented strain than in the corresponding virus-free strains when the strains were incubated for 48 and 72 h in CM liquid culture (data not shown). In a previous study, *FgHal2* was previously found to be down-regulated by FgV1 infection when we analysis the protein expression level of virus- infected and virus-free *F. graminearum* by two-dimensional electrophoresis (Kwon et al., 2009a). This down-regulation was consistently observed at the transcription level in qRT-PCR result in the current study. These results indicate that FgV1 infection affect the reduction

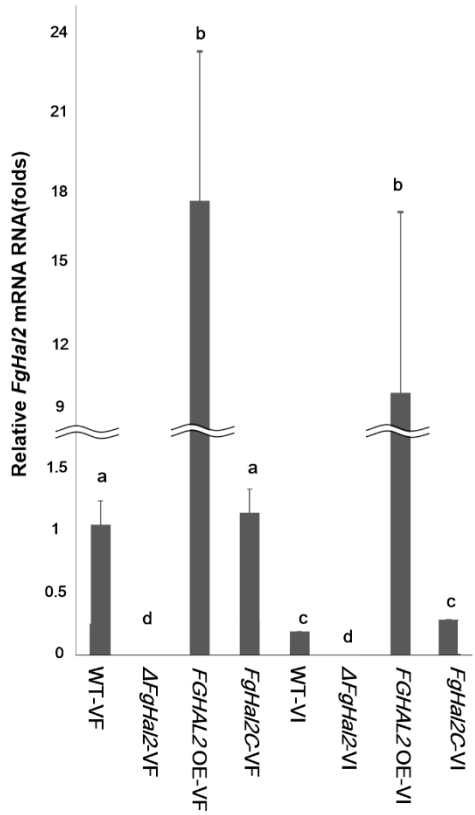

Fig. 5. Relative *FgHal2* mRNA transcript levels in the WT and mutant strains. Relative transcript levels were normalized to elongation factor 1 and ubiquitin C-terminal hydrolase. cDNAs were generated from total RNA extracts from mycelia harvested after 120 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on the Tukey test. VF, virus-free; VI, FgV1-infected strains.

of *FgHal2* expression in *F. graminearum*. *FgHal2* transcript levels in over-expression strains, however, were similar regardless of virus infection. A plausible explanation of this result is *EF1α* promoter that used for generation of the over-expression strain constitutively express the *FgHal2*.

IV. **Involvement of *FgHAL2* on vegetative growth and pigmentation**

Fungal morphology, mycelial growth were assessed to analysis the effect of *FgHal2* deletion or overexpression on morphological development in *F. graminearum*. *FGHAL2* OE-VF and *FgHal2*C-VF did not differ from that of the WT-VF morphologies on complete medium (CM) at 120 hpi, but the deletion of *FgHal2* (Δ*FgHal2*-VF) showed abnormal colony morphology, a slow growth rate, reduced aerial hyphal formation, and reduced red pigmentation (Fig. 6). The phenotypic defects of *FgHal2* could be restored by genetic complementation of *FgHal2*. Relative to the virus-free strains, the virus-infected strains had an irregular morphology, increased pigmentation, and reduced aerial mycelia at 120 hpi on CM as previously reported (Lee *et al.*, 2014). Radial growth on CM was reduced for the deletion mutant whether it was virus-free (Δ*FgHal2*-VF) or virus-infected (Δ*FgHal2*-VI). Radial growth of *FGHAL2* OE-VF and *FGHAL2* OE-VI were not different compared to WT-VF and WT-VI, respectively.

The colonies of Δ*FgHal2*-VF and Δ*FgHal2*-VI growing on CM agar showed a dramatic decrease in red pigmentation. Δ*FgHal2*-VF formed a hyphal mass that was pale yellow or white compared to that of WT, the complementation, and the over-expression strain. These results suggested *FgHal2* might plays a prominent role in the regulation and vegetative growth and pigment formation. To confirm this speculation, semi-quantitative RT-PCR analysis was performed on genes involved in the regulation of aurofusarin (AUR). *Gip2* (transcription factor), *pks12* (polyketide synthase 12), *FgOs2* (osmoregulatory MAPK), and AURj (O-methyltransferase) were not detected by qRT-PCR in Δ*FgHal2*-VF (Fig. 7A). Moreover, qRT-PCR indicated that the transcript levels of *FgOs2* and *pks12* were significantly differ in *FGHAL2* OE-VI and WT-VI compared with those of WT-VF (Fig 7B). These results support the inference that FgV1 infection and *FgHal2* might be associated with aurofusarin biosynthesis or affect osmoregulation by the MAPK-signaling pathway. Therefore, the morphological phenotype of Δ*FgHal2*-VF and the alteration of transcript accumulation of secondary metabolite synthesis genes suggest that *FgHal2* is affected to regulation of secondary metabolite production.

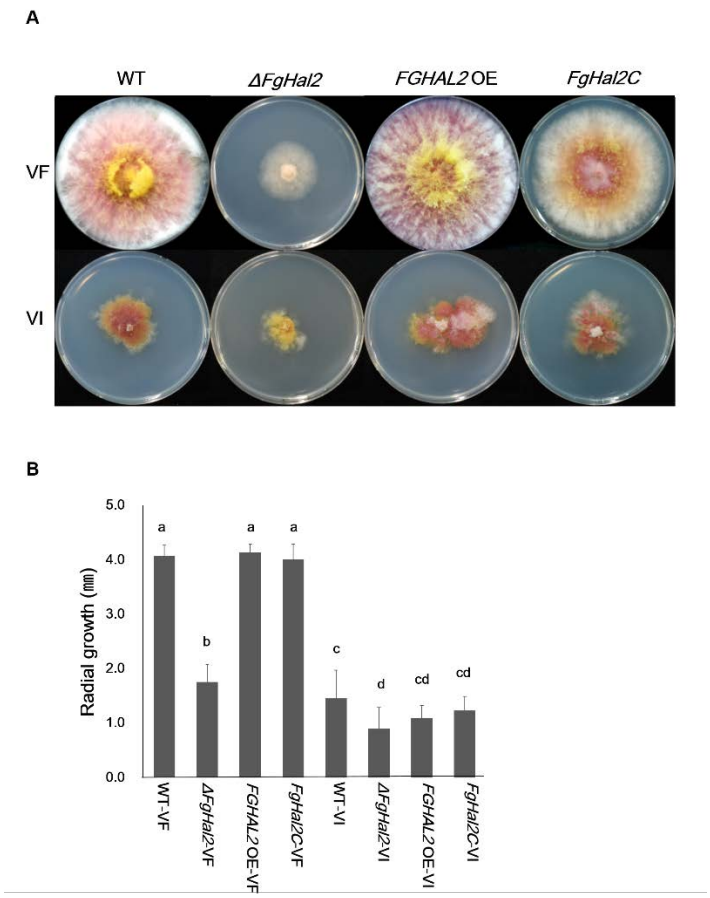

Fig. 6. Colony morphology and mycelial growth of virus-free and FgV1-infected strains (WT and *FgHal2* gene deletion, complementation, and overexpression mutants) of *F. graminearum*. (A) Colony morphology on CM agar. Colonies were photographed after 5 days of incubation. (B) Radial growth after 5 days of incubation on CM agar. Means and standard deviations were calculated from three repeated experiments. Bars with different letters are significantly different ($P < 0.05$) based on the Tukey test, which was conducted using SPSS 12.0 (SPSS, Inc., Chicago, IL).

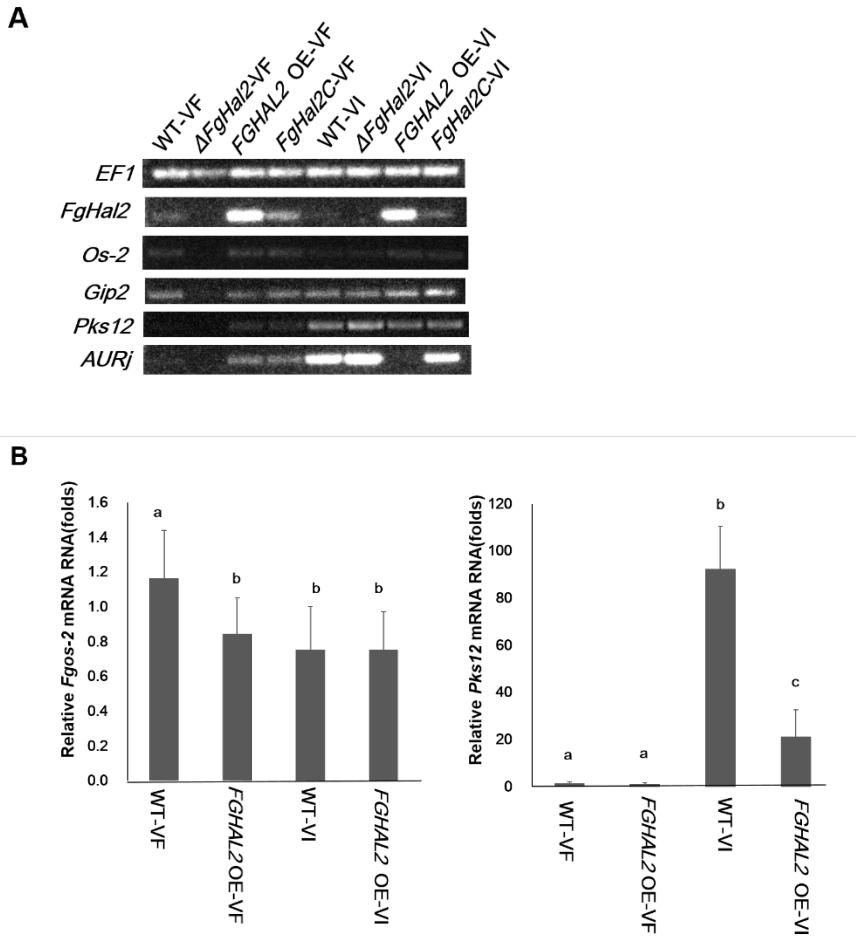

Fig. 7. Confirmation of relative expression of genes related to secondary metabolite synthesis. (A) Semi-quantitative RT-PCR of *FgOs-2*, *Gip2*, *Pks12*, *AURj*, and *FgHal2* genes using *F. graminearum* strains. cDNAs were generated from total RNA extracts obtained after 72 h of incubation. After 25 cycles, amplified DNA was analyzed by 1.5% agarose gel electrophoresis. The *EF1α* gene-specific primer set was loaded as a control. (B) Relative mRNA expression in the *FgHal2* overexpression strain. Relative transcript levels of *FgOs-2* and *Pks12* were normalized to *EF1α* and *UBH*. cDNAs were generated from total RNA extracts after 72 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on the Tukey test. VF, virus-free; VI, virus-infected.

V. Effects of *FgHal2* on hydrophobicity of the cell surface

As shown in Figure 6, both the deletion of *FgHal2* strain and FgV1 infection strains reduced the production of aerial mycelia. Aerial hyphae formation is related to the hydrophobic property which contributes to hyphal formation in many fungal species. (Jiang et al., 2011). Accordingly, it assumed that deletion of *FgHal2* or infection by FgV1 might reduce the hydrophobicity of surface of fungal colonies. To examine the hydrophobicity of the hyphal surface, 10-µl of water containing 2.5% of bromophenol blue was placed to 3-day-old colonies of virus-free and virus-infected strains (Fig. 8). The applied solution formed spherical droplets on all virus-free strains that produced abundant mycelia including the WT, the over-expression strain, and the complementation strain. In contrast, water droplets were absorbed immediately by the mycelia of the knockout mutant and of the virus-infected WT and mutant strains. These results indicate that *FgHal2*, in addition to being involved in mycelial growth, is involved in the hydrophobicity of *F. graminearum* aerial hyphae. FgV1 infection also perturb the hydrophobicity of the aerial mycelia, perhaps because FgV1 infection reduces the expression of *FgHal2*.

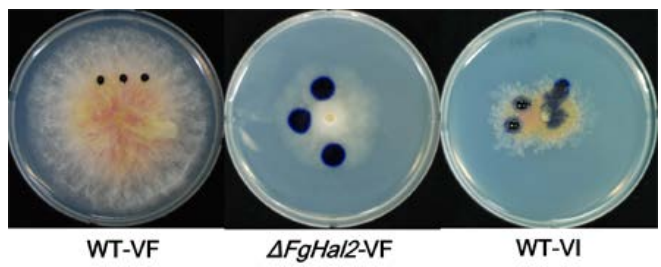

Fig. 8. Effect of *FgHal2* gene deletion on the hydrophobicity of the surface mycelium. The WT, *FgHal2* gene deletion mutant and FgV1-infected WT were grown on CM for 3 days. Each colony was treated with 15-μl of a 2.5% bromophenol blue solution. Colonies were photographed 10 min later.

VI. Involvement of *FgHal2* on conidiation and conidial morphology

To assess the production and morphology of conidia in *F. graminearum* strains, conidia production of virus-free and virus-infected strains were quantified on CMC medium (Fig. 9A). Deletion of *FgHal2* greatly reduced conidial production on CMC media regardless of FgV1 infection. Conidial production was similar among the WT, the complementation strain, or the over-expression strain regardless of FgV1 infection. Our previous study also indicated that FgV1 infection did not affect conidial production (Lee et al., 2014).

Previously, it showed that FgV1-infected *F. graminearum* produced swollen and short conidia (Lee et al., 2014). In the current study, all virus-infected strains also produced swollen and short conidia (Fig. 9B). With respect to virus-free strains, Δ*FgHal2*-VF produced shorter conidia than WT-VF, *FgHal2*C-VF, or *FGHAL2* OE-VF. Overall, these results demonstrated that the *FgHal2* gene is related to the conidial production and development.

VII. Impact of *FgHal2* on virulence of *F. graminearum*

To assess the effect of *FgHal2* on *F. graminearum* virulence, conidial suspensions of virus-free and virus-infected WT *F. graminearum* strains were inoculated on wheat heads. Fusarium head blight symptoms appeared on

wheat heads inoculated with the WT-VF, *FGHAL2* OE-VF, and *FgHal2*C-VF (Fig. 10A). However, head blight symptoms were not noted on wheat head that inoculated with Δ*FgHal2*-VF. Because growth rate of Δ*FgHal2*-VF showed severely decreased relative to that of the WT, Δ*FgHal2*-VF was monitored inoculated wheat heads for a prolonged period, however, no symptoms were observed even at 21 days post-inoculation. All the virus-infected strains were not detected head blight symptoms the wheat heads or on the inoculated spikelets (Fig. 10B)

VIII. Involvement of *FgHal2* on transmission of FgV1

To determine whether *FgHal2* affect the stability of the FgV1 in *F. graminearum*, vertical transmission of FgV1 through the asexual spores of the WT and the mutant strains were evaluated (Table 4). The WT-VI and the *FgHal2*C-VI show 100% of transmission ratio in all generation. The *FGHAL2* OE-VI was slightly reduced in the first generation but was 100% for the second and third generation. The transmission of Δ*FgHal2*-VI reduced considerably with in process of generation. These data indicate that interfere vertical transmission of FgV1 via conidia. Also, it is possible that deletion of the *FgHal2* gene disturb the maintenance of FgV1 in infected host cells.

To assess the stability of FgV1 in mycelia in *F. graminearum*, FgV1

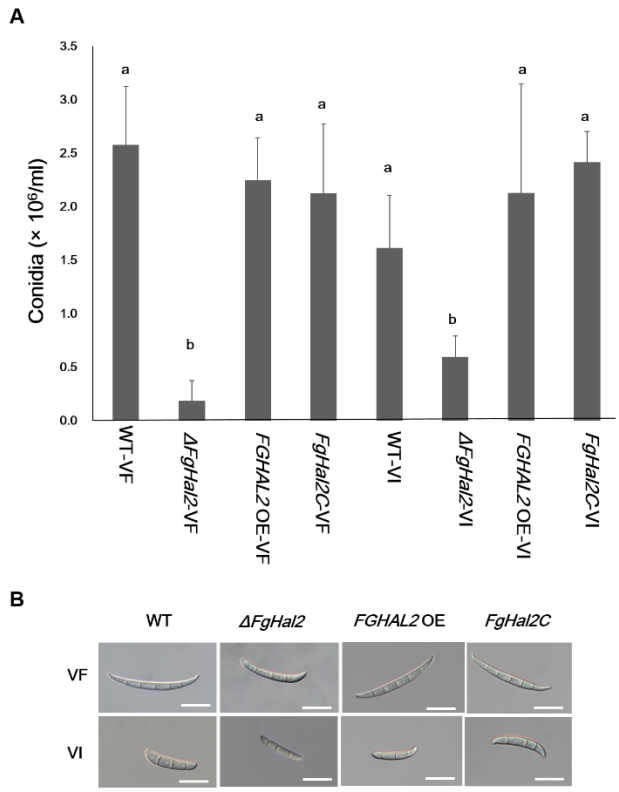

Fig. 9. Conidial production by virus-free and FgV1-infected strains (WT and *FgHal2* gene deletion, complementation, and overexpression mutants) of *F. graminearum*. (A) Conidia production after 5 days on CMC. Values are means and standard deviations of three repeated experiments. Means with different letters are significantly different ($P < 0.05$) based on the Tukey test. (B) Conidial morphology. Representative conidia of virus-free (VF) and virus-infected (VI) strains were photographed with differential interference contrast optics. Bar = 20 μm.

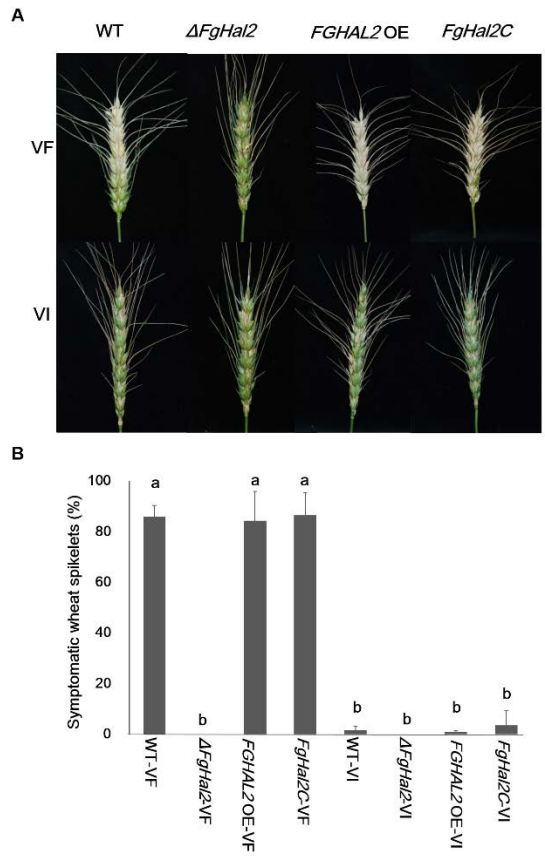

Fig. 10. Virulence of virus-free and FgV1-infected *F. graminearum* strains (WT and *FgHal2* gene deletion, complementation, and overexpression mutants) on wheat. (A) Representative wheat heads 14 days after they were inoculated with conidia. (B) The percentage of symptomatic wheat spikelets 14 days after inoculation. Means and standard deviations were calculated from independent, repeated experiments. Error bars indicate standard deviation. Bars with different letters are significantly different ($P < 0.05$).

infected strains were subculture onto PDA (Fig.11). During the repeated subculturing on PDA media, the WT-VI, *FgHal2*C-VI, and *FGHAL2* OE-VI did not produce virus cured isolate under typical condition. Unlike these strains, Δ*FgHal2*-VI rapidly produced a colony with virus free and virus-infected sectors. Also, the phenotype of transferred Δ*FgHal2*-VI showed different among Δ*FgHal2*-VI strains. These result indicated that *FgHal2* is related to the virus transmission of FgV1 in *F. graminearum*.

IX. Effect of *FGHAL2* on accumulation of FgV1 RNA

qRT-PCR was carried out to analyze the influence of *FgHal2* on viral-RNA synthesis (Fig. 12). At 48 hpi, viral RNA levels were significantly greater in the WT than in the Δ*FgHal2*-VI, *FGHAL2* OE-VI, or *FgHal2*C-VI. At 72 hpi, viral RNA levels had remarkably increased in all strains except Δ*FgHal2*-VI. At 120 hpi, viral RNA levels remained low in the Δ*FgHal2*-VI. Interestingly, RNA accumulation of *FGHAL2* OE-VI had greatly decreased but that of WT-VI and the *FgHal2*C-VI had dropped only moderately at 120 hpi. These results suggest that *FgHal2* may affect FgV1 RNA accumulation either directly or indirectly.

Table 4. Vertical transmission of FgV1 between generations of *Fusarium graminearum* strains.

	Vertical transmission rate (%)		
Strain	1st generation	2nd generation	3rd generation
WT-VI	100	100	100
Δ*FgHal2*-VI	63	45	15
FGHAL2 OE-VI	84	100	100
*FgHal2*C-VI	100	100	100

Vertical transmission was measured as the percentage of FgV1-positive isolates among the total number of single-conidium isolates. Each value is based on > 30 single-conidium isolates.

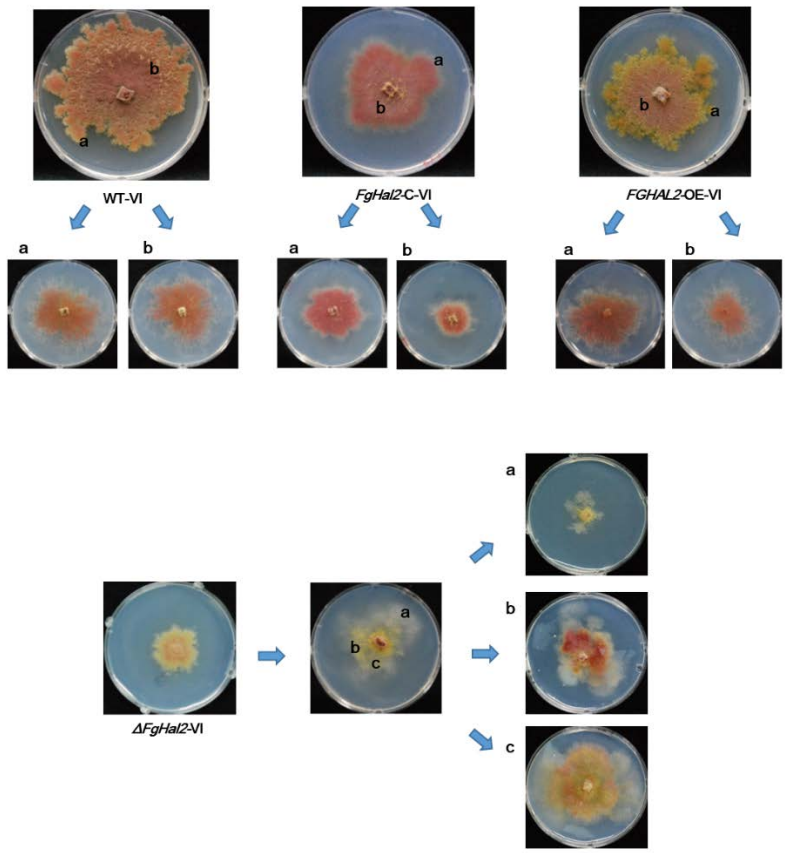

Fig. 11. The effects of subculturing on morphology of virus-infected *F. graminearum* strains growing on PDA. Single spores of all virus-infected strains were inoculated on PDA containing appropriate antibiotics. After 5 days, a mycelial plug from each plate was transferred to fresh PDA. Δ*FgHal2*-VI was transferred once more and photographed 5 days later.

X. Effect of *FgHal2* on silencing related genes

FgHal2 ortholog genes in *Arabidopsis* and yeast, *FRY1* and *Met22*, respectively, convert pAp which inhibit ribonuclease activity of Xrn. Xrn activities are repressed in FRY1 mutant, leading to increase RNA silencing triggers (Gy et al., 2007). Accordingly, it was hypothesized that *FgHal2* is probably associated with RNA silencing suppression in *F. graminearum*.

Previous research showed the variation in post-transcriptional gene silencing (PTGS)-related gene expression by qRT-PCR (Lee et al., 2014). Among the argonaute-like genes (*agl*) and dicer-like genes (*dcl*) in *F. gramimearum*, *dcl1* (FGSG_09025) was significantly up-regulated by FgV1 infections whereas expression of *agl2* (FGSG_08752) was down-regulated by FgV1 infection (Lee et al., 2014). In this study, the gene expression level of *dcl1* and *agl2* were examined to confirm whether expression level of PTGS-related gene is changed by deletion or overexpression of *FgHal2* (Fig. 13). *Dcl1* transcript level was strongly increased in Δ*FgHal2*-VF and all FgV1-infected strains. Also, *agl2* gene expression level was up-regulated in Δ*FgHal2*-VI, however, level of WT-VI, *FgHal2*C-VI, and *FGHAL2* OE-VI were slightly decreased as compared to those of virus-free strains. Presumably, similar with *Hal2* orthologs, it is possible that gene deletion of *FgHal2* lead to induction of PTGS requiring *dcl1*.

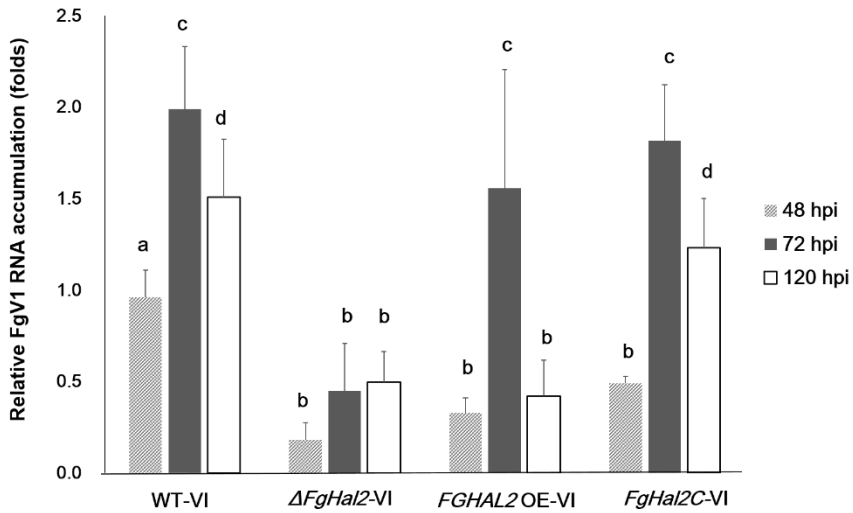

Fig. 12. Accumulation of FgV1 viral RNA in FgV1-infected WT, Δ*FgHal2*, *FgHal2C*, and *FGHAL2* OE strains of *F. graminearum*. qRT-PCR was used to quantify FgV1 viral RNA at 48, 72, and 120 hpi. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on the Tukey test.

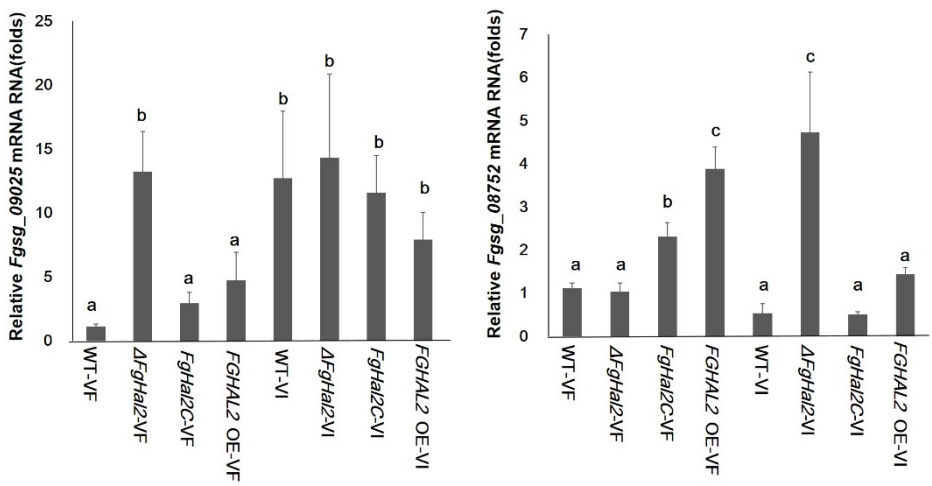

Fig. 13. Accumulation of silencing relate genes in *F. graminearum* strains Relative mRNA expression of *dcl1* and *agl2* genes in the *F. graminearum* strains. Relative transcript levels of *dcl1* (FGSG_09025) and *agl2* (FGSG_08752). Relative transcript levels were normalized to *EF1α* and *UBH*. cDNAs were generated using total RNA extracts from mycelia that were collected after 120 h of incubation. Error bars indicate standard deviations. Values with different letters are significantly different ($P < 0.05$) based on the Tukey test. VF, virus-free; VI, virus-infected.

DISCUSSION

In computational sequence analysis, *FgHal2* was predicted to encode a 3'-nucleotidase that is highly conserved in fungi. This nucleotidase is a member of the family of phosphatases that share the properties of sensitivity to Li^+ and Na^+ ions, metal-dependency, and substrate selectivity (Hudson and York, 2012). *S. cerevisiae* has 3'-nucleotidase gene, but two or more genes codes for this enzyme in other fungi and plants including *Arabidopsis*. The isoforms of pAp phosphatase enzymes are differentially regulated during organ differentiation and stress responses and such enzymes usually exhibit substrate stringency and sensitivity to toxic cations (Gil-Mascarell et al., 1999; Hudson and York, 2012). *F. graminearum* has three genes with pAp phosphatase-conserved domains. Interestingly, only *FgHal2* was significantly down-regulated by FgV1 infection (Fig. 2). This result suggests that *FgHal2* might be closely involved in certain regulatory signal processes that response to by FgV1 infection.

As mentioned above, *Hal2* (*Met22*) has been identified and characterized in yeasts (Gläser et al., 1993; Vaupotič et al., 2007) and plants (Gil-Mascarell et al., 1999). However, the function of this gene in plant-pathogenic fungi has not been determined. Because inhibition of *Hal2* causes PAP accumulation in

the cell, it also inhibit sulfotransferase activity, RNA-processing enzymes, and the sulfur assimilation pathway (Gašparič et al., 2013; Belles and Serrano, 1995). Accordingly, these lead to impede growth. FRY1 in *Arabidopsis* is a homolog of MET22 in yeast, and the *fry1* mutant generated fewer lateral roots and had stunted growth, short hypocotyls, curly leaves, and a retarded phase transition and leaf initiation (Chen and Xiong, 2010). In this study, deletion of the *FgHal2* gene significantly reduced the growth of embedded and aerial hyphae on the agar media. Deletion of *FgHal2* also reduced conidial production and resulted in abnormal conidial morphology. These results suggest that *FgHal2* is involved in the regulation of *F. graminearum* growth and development. In addition, deletion of *FgHal2* or FgV1 infection lead to reduce hydrophobicity of the fungal colony, suggesting that *FgHal2* contributes to cell wall integrity and reduced *FgHal2* expression upon FgV1 infection might related to the morphological phenotype of virus-infected fugal strain.

F. graminearum produces diverse secondary metabolites including the polyketide pigments aurofusarin (AUR) and rubrofusarin (Kim et al., 2006). To date, few genes have been identified that participate in the biosynthesis of AUR (Geng et al., 2014). Gip2 is a putative positive regulator of the AUR biosynthetic gene cluster, and polyketide synthase 12 (pks12) is upstream of

Gip1 and is responsible for changing a precursor to AUR (Geng et al., 2014). In Fig. 6, Δ*FgHal2*-VF and Δ*FgHal2*-VI greatly reduced red pigmentation on CM agar media. Also, no red pigment accumulated in Δ*FgHal2*-VF and Δ*FgHal2*-VI, even when cultured on PDA plates for 7days (data not shown). This result was agreement with the dramatically reduced expression level of pigmentation-related genes (*Gip2*, *pks12*, *FgOS2*, and *AURj*) in Δ*FgHal2*-VF (Fig. 7). However, *pks12* and *AURj* greatly increased by FgV1 infection although *FgHal2* transcript level was reduced. It is possible that another signaling or pathway is also involved in this secondary metabolite production because fungi have a complex regulatory mechanism to produce a secondary metabolites and FgV1 can interact with broad range of molecules in this metabolism pathway. Additionally, *FgOS2* significantly reduced the transcript level in *FGHAL2* OE-VF compared with that of WT-VF and *pks12* expression was also reduced approximately 70% in *FGHAL2* OE-VI compared with that of WT-VI (Fig. 7). This result indicated that overexpression of *FgHal2* also affect the expression of pigmentation-related genes. Also, unlike gene deletion mutant, *FgHal2* was not completely suppressed in WT-VI, the semi-qRT-PCR result still support the relationship between *FgHal2* and secondary metabolism production.

An osmosensor histidine kinase (*FgOs1*) and an osmoregulatory MAPK

pathway (consisting of *FgOs4*, *FgOs5*, and *FgOs2*) are associated with the regulation of AUR in *F. graminearum* (Ochiai et al., 2007). The current study demonstrated that FgV1 infection and *FgHal2* might regulate aurofusarin biosynthesis so as to affect osmoregulation by the MAPK-signaling pathway. The distinguishable morphological phenotype of Δ*FgHal2*-VF and the alteration of transcript levels of secondary metabolite synthesis genes in *FGHAL2* OE and WT-VI suggest that *FgHal2* is involved in secondary metabolite production. Thus, suppression of *FgHal2* in WT-VI might be involved in the biological and physiological changes caused by FgV1 infection. Probably, mycovirus infection and fungal secondary metabolism are sharing multiple regulatory pathways. Previous research showed that CHV1-EP713 hypovirus modulates host G-protein signal transduction (Dawe et al., 2004) and the *Hog1*-homologue *cpmk1* gene of the MAPK pathway of *C. parasitica* (Park et al., 2004). These signaling pathways are associated with regulation of secondary metabolite synthesis. In this regard, the expression levels of genes in the osmotic stress signal transduction pathway (*FgOs5* and *FgOs2)* and those related to polyketide synthase (*Pks12*, *Gip4*, and *Gip5*) were significantly different in the FgV1-infected WT than in the virus-free WT (Lee et al., 2014). It would help to determine how *FgHal2* affects secondary metabolite synthesis pathways that might be associated with FgV1

infection.

In qRT-PCR results for FgV1 RNA accumulation, the FgV1 RNA level in *FGHAL2* OE-VI was low relative to that in WT-VI at 48 hpi, increased at 72 hpi, and then returned to a low level at 120 hpi (Fig. 12). The *FgHal2* expression level, however, *FGHAL2* OE-VI was approximately 9-fold higher than in WT-VI. A possible explanation for this decrease in *FGHAL2* OE-VI is that potential role(s) in *FgHal2*. Over-expression of *FgHal2* might assist the fungus overcome osmotic and other stresses, as has been demonstrated for the ortholog of *FgHal2*, *Hal2* (Gašparič et al., 2013; Shinozaki and Yamaguchi-Shinozaki, 1999). As noted earlier, the small heat-shock protein CpHsp24 contributes to stress adaptation but not to hypoviral replication in *C. parasitica* (Baek et al., 2014). If hypovirulence-associated FgV1 infection is considered a biological stress to *F. graminearum*, fungal stress-responsive pathways or elements may also be affected by the virus infection. This result proposed that *FgHal2* might affect host defense response. Further study will be needed to discover correlation between *FgHal2* and stress-responsive pathways in *F. graminearum*.

In qRT-PCR, because *FgHal2* was repressed by FgV1 infection, it was assumed that the viral RNA accumulation level would be similar or higher in virus-infected Δ*FgHal2* than in the virus-infected WT. However, FgV1 RNA

accumulation was significantly less in Δ*FgHal2*-VI than in WT-VI. This result indicate that the *FgHal2* gene may also directly or indirectly affect virus RNA accumulation. Perhaps reduced expression level of *FgHal2* in response to virus infection does not protect the fungal host in some respects but does generate unsuitable conditions for FgV1 persistence in host cells.

It was reported that repressed Xrns activity leads to increased accumulation of aberrant RNAs that trigger post-transcriptional gene silencing (Gy et al., 2007). Also, TBSV RNA recombination occurs in high frequency when the activity of Xrn1p is inhibited by elevated pAp/pNp levels due to the deletion of *Met22* gene (Jaag and Nagy, 2010). As shown in Fig.13, deletion of *FgHal2* and FgV1 infection lead to greatly increase the accumulation of *dcl-1*, this accumulation level is similar to virus-infected strain. This result suggest the possible role of *FgHal2* in host defense response against the FgV1 infection. Also, this result might be one of reasons low accumulation of FgV1 by trigger PTGS. It is remained that to provide correlation among dicer, *FgHal2*, and FgV1 and antiviral defense response in *F. graminearum* in further study.

The FgV1 transmission experiment in the present study suggests that *FgHal2* may support virus transmission. Relative to the vertical transmission in the FgV1-infected WT, the vertical transmission in Δ*FgHal2*-VI decreased

gradually and was only 15% in the 3rd generation (Table 4). This failure to maintain FgV1 was also observed during serial subcultures of mycelia on agar media. Δ*FgHal2*-VI seems rapidly lost FgV1 in partial region of colony and produced virus free and virus-infected sectors during the repeated subculture onto PDA. These observations demonstrated that *FgHal2* is necessary for the maintenance of FgV1 infection. The *C. parasitica pro1* gene, which encodes the $Zn(II)_2Cys_6$ transcription factor, showed similar phenomenon. The hypovirus-infected *pro1*-disruption mutant was frequently recovered from hypovirus and produced the virus free sector and virus-infected sectors on colony (Sun et al., 2009). *FgHal2* and *pro1* levels were down-regulated upon virus infection but not eliminated. These results may explain why *F. graminearum* down-regulates *FgHal2* expression after FgV1 infection. The decrease in vertical transmission and low stability after successive subcultures on agar for the *FgHal2* deletion mutant indicate that FgV1 requires *FgHal2* to maintain itself in the host. Therefore, down-regulation of the expression of *FgHal2* gene is harmful for the fungal host but this reduction might be inevitable consequence for host to disturb the persistence of virus and to induce the defense-response. However, the association between *FgHal2* and maintenance of FgV1 infection needs further investigation.

In conclusion, our results suggest that putative 3'(2'), 5'-bisphosphate

nucleotidase gene *FgHal2* is necessary for vegetative growth, conidial production, conidial maturation, and secondary metabolite synthesis in *F. graminearum*. However, *F. graminearum* might represses *FgHal2* expression to reduce FgV1 viral RNA accumulation and transmission by generate unstable conditions for the maintenance of FgV1. Biochemical analyses of the *FgHal2* is needed to understand the specific function of *FgHal2* in *F. graminearum*. Moreover, further studies will be expected to elucidate the mechanistic aspects of processes that involved in the interaction between *FgHal2* and FgV1 in greater detail.

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붉은 곰팡이에 감염하는 바이러스의 분자 생물학적 특성 및 방어 반응 관련 기주 요인의 특성 구명

유지숙

초록

Fusarium graminearum 을 비롯한 *Fusarium* 속의 식물병원성 곰팡이에 감염하는 바이러스들이 수 차례 보고되고 있다. *F. graminearum* 에 감염하는 대부분의 곰팡이 바이러스 들은 기주의 형질에 아무런 영향을 끼치지 않은 채로 기주 내에 영구적으로 존재한다. 그러나, 몇몇 바이러스 들은 곰팡이에 감염하여 생장속도의 저하와 색소 생성의 증가, 포자형성 감소, 병원성 감소 등의 표현형의 변화를 일으킨다. *F. graminearum* 에 감염하는 바이러스 중에, FgV1 과 FgV2 는 균주에 감염 시에 기주의 저병원성 (hypovirulence) 의 병징에 부합하는 특징을 나타낸다. 반면, FgV3 와 FgV4 의 감염은 어떠한 눈에 띄는 형질의 변화를 일으키지 않는다. 본 연구를 통해, FgV2, FgV3, FgV4 의 유전자 게놈 서열을 밝히고 이를 바탕으로 기존에 보고된 여타의 곰팡이 바이러스들과 계통유전학적 분석을 통해 그 관계를 밝히고자 한다. FgV2, FgV3, FgV4 는 하나의 게놈을 가진 것부터 다섯 개의 double-strand 절편을 가진 바이러스로 분석되었고, 게놈의 크기는 최소 1.7 kb 에서 최대 9.3 kb 의 게놈을 가지고 있는 것으로 확인되었다. FgV2 의 경우, monocistronic dsRNA segments 를 가지고 있으며 전체 게놈 사이즈가 2414 bp 에서 3580 bp 사이의 절편으로 구성되어 있었다. FgV3 9098 bp 의 단가닥 바이러스로 잘 보존된 RdRp domain 을 포함한 두 개의 ORF 로 구성되어 있다. FgV4 는 두 가닥의 절편으로 나뉘어 있으며, 크기는 2383bp 와 1739bp 로 분석되었다. RdRp domain 을 포함한 부분의 계통 유전학적 분석을 통해 FgV2, FgV3, FgV4

는 *Chrysoviridae*, *Totiviridae, Partitiviridae* 등과 각각 가까운 유연관계가 있지만 그 그룹 안에서는 아직 분류되지 않은 곰팡이 바이러스들과 또 다른 하위 그룹을 구성하고 있는 것으로 분석되었다.

전사체와 단백질체의 발현 비교 연구 결과, 많은 수의 *F. graminearum* 의 유전자들이 바이러스 감염 결과 발현 양상이 달라지는 것을 확인하였다. 이러한 유전자들 중에서는 곰팡이 바이러스와 기준 간에 상호 작용을 통해 바이러스의 생활사를 완성하는 데 필수적인 요소들과 바이러스 감염에 따른 기주의 방어기작과 관련한 유전자들도 포함되어 있을 것이다. 이렇게 FgV1 감염 시 발현 양상이 달라지는 유전자 중, *FgHal2* 는 FgV1 감염 후 그 발현량이 줄어드는 것으로 관측되었다. *FgHal2* 는 yeast, Arabidopsis와 다른 filamentous fungi들 감에 비교적 잘 보존되어 있는 3'-phosphoadenosine 5'-phosphatase (PAP phosphatase-like) domain 또는 inositol monophosphatase (IMPase) domain 을 가지고 있는 것으로 확인되었다. 바이러스와의 연관성과 더불어 균주 내에서의 자세한 기능 분석을 위해 해당 유전자를 불활성화시킨 변이체와 과발현시킨 변이체를 제작하였다. *FgHal2* 유전자의 삭제는 표현형 상에서 균사생장 저하와 공중 균사생성 불능, 색소의 감소 등을 일으켰으나 과발현 균주의 경우에는 wild-type과의 차이가 없었다. 더불어, 해당 유전자의 삭제는 conidia의 생성과 비정상적인 형태의 conidia를 상대적으로 많이 생성하는 것을 확인하였다. 바이러스에 감염된 균주 내에서 *FgHal2* 유전자의 발현은 접종 120시간 이후에 건전 기주에 비해 감소함을 확인하였고, 과발현 균주에서는 건전 기주에 비해 바이러스의 감염 여부와 상관없이 약 9개 가량 증가해 있는 것을 확인하였다. 접종 후 48, 72, 120 시간 이후 FgV1 RNA의 발현량을 확인해본 결과, deletion mutant 에서는 시간이 지나도 대조군에 비해서 발현량이 현저하게 감소하여 있는 것을 확인하였다. 반면, *FgHal2* 과발현 균주의 경우, 접종 후 72시간 까지는 바이러스에 감염된 일반 균주와 비슷한 발현 양상을 보였지만, 120시간 째에는 눈에 띄게 감소하는 양상을 나타내었다. 무성포자를 통한 바이러스의 전이 효율을 확인해본 결과 유전자를 삭제한 균주에서 그 전이율이 낮아짐을 확인하였고, 계대 배양 시에 한 콜로니 내에서 바이러스에 감염된 부분과 회복된 부분이 공존하는 경우가 많은 것을 확인하였다. 이러한 일련의 결과는

FgHal2 가 FgV1이 기주 내에서 안정적으로 생존할 수 있도록 하는 기능과 관련성이 있음을 말해준다. 결론적으로, 3'(2'), 5'-bisphosphate nucleotidase를 암호화하는 유전자를 가진 *FgHal2*는, 붉은 곰팡이 내에서 생장과 이차 대사산물 생성을 비롯한 다양한 기능과 연관성이 있으며, FgV1 바이러스의 복제와 전이에 대한 관련성을 가지고 있으리라 여겨진다.

KEY WORDS: 붉은 곰팡이, 곰팡이 바이러스, *FgHal2*, 3'(2'), 5'-누클레오티드가수분해효소, 곰팡이-바이러스 상호작용